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IN-SITU BIOREMEDIATION OF SOLVENT SATURATED SOILS USING METHANE, PROPANE, AND BUTANE-OXIDIZERS

LEWIS SEMPRINI
ADISORN TOVANABOOTR
PARDI JITNUYANONT

DEPT OF CIVIL, CONSTRUCTION,
AND ENVIRONMENTAL ENGINEERING
OREGON STATE UNIVERSITY
CORVALIS OR 97331

2 FEBRUARY 2000

AIR FORCE RESEARCH LABORATORY
MATERIALS & MANUFACTURING DIRECTORATE
AIRBASE & ENVIRONMENTAL TECHNOLOGY DIVISION
TYNDALL AFB FL 32403-5323

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Alison Lightner

ALISON LIGHTNER
Program Manager

Christine Wagener-Hulme

CHRISTINE WAGENER-HULME
Lt Col, USAF, BSC
Chief, Environmental Technology
Development Branch

Christine Wagener-Hulme for

RANDY L. GROSS, Col, USAF, BSC
Chief, Airbase and Environmental
Technology Division
acting Chief

Andrew D. Poulis

ANDREW D. POULIS
STINFO Program Manager

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Lewis Semprini, Adisorn Tovanabootr, and Pardi Jitnuyanont

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The focus of this work was to evaluate the potential of aerobic microorganisms, grown on butane propylene, and propane, to cometabolically degrade chlorinated aliphatic hydrocarbons (CAHs). The key contaminant of concern was trichloroethylene (TCE). However, other CAHs of interest were chloroform (CF), 1,1,1-trichloroethane (TCA), 1,1-dichloroethylene (1,1-DCE). The study focused on microbes stimulated from the subsurface core materials from Air Force Base (AFB) sites with subsurface CAH contamination. Comparisons were also made with other gaseous substrates such as methane, propylene, and ammonia that have shown activity for TCE cometabolism. Through microcosm and soil column studies the work determined the potential for applying these processes for in-situ remediation of CAH contamination at AFB sites.

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Chlorinated aliphatic hydrocarbons, CAHs, trichloroethylene, TCE, chloroform, 1,1,1-trichloroethane, TCA, 1,1-dichloroethylene, 1,1-DCE, methane, propylene, and ammonia, microcosm, subsurface, gaseous, cometabolism, in situ remediation

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PREFACE

This report was prepared by Oregon State University, Department of Civil, Construction, and Environmental Engineering, Corvalis, OR 97331, for the US Air Force Research Laboratory, Materials and Manufacturing Directorate, Airbase and Environmental Technology Division (AFRL/MLQE), Suite 2, 139 Barnes Drive, Tyndall Air Force Base, Florida 32403-5323.

The focus of this work was to evaluate the potential of aerobic microorganisms, grown on butane propylene, and propane, to cometabolically degrade chlorinated aliphatic hydrocarbons (CAHs). The key contaminant of concern was trichloroethylene (TCE). However, other CAHs of interest were chloroform (CF), 1,1,1-trichloroethane (TCA), 1,1-dichloroethylene (1,1-DCE). The study focused on microbes stimulated from the subsurface core materials from Air Force Base (AFB) sites with subsurface CAH contamination. Comparisons were also made with other gaseous substrates such as methane, propylene, and ammonia that have shown activity for TCE cometabolism. Through microcosm and soil column studies the work determined the potential for applying these processes for in-situ remediation of CAH contamination at AFB sites.

This work was performed between June 1995 and March 1997. The AFRL/MLQE project officer was Ms. Alison Thomas.

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Executive Summary

The potential for using gaseous cometabolic substrates (methane, propane, butane, and propylene) for the aerobic transformation of chlorinated aliphatic compounds (CAHs), such as trichloroethylene (TCE), was evaluated in this work with microorganisms stimulated from DOD sites. Three DOD sites were evaluated for potential demonstrations of aerobic cometabolism using these gaseous substrates. The McClellan Air Force Base, CA; Edwards Air Force Base, CA; and the Moffett Air Field, CA. The ability to stimulate indigenous microorganisms from these sites on these gaseous substrates was determined, as well as their effectiveness at transforming CAHs of interests. The CAHs studied in the most detail were TCE, 1,1,1-trichloroethylene (1,1,1-TCA), and chloroform (CF), which are representative of chlorinated ethenes, ethanes, and methanes frequently observed as DOD subsurface contaminants.

Initial surveys helped us determine the microbial systems and processes on which to focus our work. The initial surveys show indigenous microorganisms could be rapidly stimulated in groundwater and in soil microcosms from the McClellan subsurface. Microorganisms were most rapidly stimulated on methane, followed by propane, butane, and propylene. Microorganisms stimulated on methane and propane were found to be most effective in transforming TCE. Propylene-utilizers showed limited ability to transform TCE, and butane-utilizers showed no ability. In Edwards microcosms, methane-utilizers were easily stimulated that transformed TCE. Propane and butane-utilizers were not stimulated over a 90-day period. The results indicated that these microorganisms were not likely present in the Edwards subsurface. TCE transformation was observed to be less effective by methane-utilizers stimulated from the Edwards site compared to the McClellan site. At the Moffett Field site microcosms studies showed a very long lag period (over 80 days) for stimulation on propane or butane. These studies focused on 1,1,1-TCA transformation, and showed initially limited ability of the propane and butane-utilizers to transform 1,1,1-TCA.

The results of the initial screening studies indicated that indigenous methane and propane-utilizers where present in the McClellan subsurface that had potential for TCE transformation. Thus we chose to study these systems in detail. The second important observation is that propane and butane-utilizers that effectively transform CAHs are not always present at sites, or may require long lag periods to biostimulate effective microorganisms. Thus bioaugmentation of the subsurface may be required. We therefore investigated bioaugmentation approaches for the Moffett Field site.

The Moffett Field studies evaluated biostimulation approached for aerobic cometabolism of 1,1,1-TCA. Long lag periods (80 to days) were observed before butane or propane uptake was observed in microcosms. The results indicated that indigenous propane or methane-utilizers where in very low numbers in the subsurface or may have been introduced into the microcosms during their long-term operation. Mixed cultures were augmented into one set of microcosms after 55 days of stimulation. In the butane bioaugmented microcosm, butane uptake was observed within a few days of bioaugmentation, and 1,1,1-TCA was transformed. The microcosm that was not augmented required 80 days of incubation before butane was consumed, and 1,1,1-TCA was not transformed. The results indicated the

bioaugmentation with butane enrichment was successful. In the propane bioaugmented microcosm, a long lag of about 25 days was observed after bioaugmentation, and propane was consumed at 80 days in the bioaugmented and 90 days in the unaugmented microcosm. In both microcosms some 1,1,1-TCA was transformed. Thus in the propane system both the augmented and unaugmented systems showed similar behavior. Indigenous microorganisms may have been stimulated in both microcosms.

The butane and propane microcosms were operated for 420 days in sequential batch operation. 1,1,1-TCA concentrations were gradually increased, while the amount of butane or propane fed was held constant. All microcosms continued to degrade 1,1,1-TCA over the entire period. The microcosm with the bioaugmented butane-utilizers initially was most effective, but by the end of the study both the augmented and unaugmented microcosms achieved the same transformation yields of about 0.07 mg 1,1,1-TCA/mg butane fed. The propane-utilizers, both augmented and unaugmented were more consistent in their transformation abilities, and showed a similar transformation performance throughout the study. By the end of the study, the transformation yields of 0.08 mg 1,1,1-TCA/mg propane were achieved. PCR analysis of the mixed cultures performed at the end of the studies showed similar fingerprints in both the butane and propane augmented and unaugmented systems. Based on the PCR results, we can not conclude that the mixed cultures differ in the augmented and unaugmented systems. Similar transformation abilities were also achieved during these periods, indicating the cultures did not differ in their transformation abilities. One possibility is that indigenous microorganisms were selected over the long period of operation of the bioaugmented microcosms.

Later attempts to bioaugment Moffett microcosms with butane or propane-utilizers failed. One possibility was the enrichments used for bioaugmentation adapted to nutrient rich media grown conditions. To test this concept bioaugmentation studies were performed with groundwater, aquifer solids, and with varying amounts of nutrient media added. For both butane and propane systems the microcosms with 50 % groundwater and 50 % media performed the best, and effectively transformed increasing 1,1,1-TCA concentrations. The microcosms fed groundwater (no media), performed the worst, and eventually lost 1,1,1-TCA transformation ability as 1,1,1-TCA concentrations were increased. The microcosm fed 95% groundwater and 5% media also lost 1,1,1-TCA transformation ability. These groundwater and 5% media microcosms also consumed butane more slowly than the 50% media and 50 % groundwater microcosms. PCR results showed different fingerprints for the microcosms fed different nutrient formulations. The differences were related more to the nutrient conditions than whether or not the microcosms were exposed to 1,1,1-TCA. Both the butane and the propane systems showed similar results. Upon regrowth on a nutrient rich media all the systems, even those that had lost 1,1,1-TCA transformation abilities, were able to transform 1,1,1-TCA.

The studies then focused on developing enrichments that grew well and transformed 1,1,1-TCA under the nutrient limited conditions of the Moffett groundwater. Enrichments for bioaugmentation were obtained from the original microcosms that were operated for 420 days. After being grown once on media they were bioaugmented into Moffett groundwater and aquifer material. Both butane and propane-utilizers effective grew and transformed

increasing concentrations of 1,1,1-TCA. The results indicate that effective bioaugmentation might be achieved through the addition of enrichments that perform well under the nutrient conditions of the groundwater.

Subsurface microorganisms from McClellan Air Force Base were grown in batch aquifer microcosms fed methane, propane, and butane, and were tested for TCE cometabolism potential. The batch microcosms consisted of aquifer solids, site groundwater, and an air filled headspace. Rapid stimulation of microbes on all of the substrates tested indicated a diverse microbial community exists in the McClellan subsurface. The lag periods of 2 weeks before active substrate consumption for methane and 3 weeks for propane and butane were observed. Methane and propane-utilizers were active toward TCE cometabolism, while butane-utilizers showed no ability to transform TCE. The mass of TCE added was gradually increased while the mass of growth substrate added was held essentially constant. TCE was successively transformed in the in the methane and propane fed microcosms for up to 1 year, but the propane-utilizers performed more poorly at high TCE concentrations. TCE was transformed most rapidly during the period of active methane consumption, and continued at a slower rate for about 1 week after methane was consumed. The propane culture remained active for up to four weeks after propane was consumed, and the rate followed first order kinetics. Different TCE transformation yields developed in replicate microcosms with time. Changes in TCE transformation ability resulted from changes in TCE concentration and/or TCE product toxicity. Both methane and propane-utilizers shows linear correlation between initial TCE transformation rates and primary substrate utilization rates. The correlation between the ratio of zero order TCE transformation rates to primary substrate utilization rates were directly proportional to transformation yields. The ratio of the rates represented about 50 % of the ultimate transformation yield for methane-utilizers, and 20 % of the transformation yield for propane-utilizers. The maximum observed TCE transformation yields were 0.068 g TCE/g methane and 0.048 g TCE/g propane.

The potential for aerobic cometabolism of chlorinated aliphatic hydrocarbons mixtures of trichloroethylene (TCE), 1,1,1-trichloroethane (1,1,1-TCA), and chloroform (CF) was determined in McClellan microcosms. Indigenous methane-utilizers were capable of transforming TCE and CF, but not 1,1,1-TCA. Propane-utilizers very effectively transformed 1,1,1-TCA, CF, and TCE. The butane-utilizers were not able to degrade any of the CAHs tested. Propane-utilizers exhibited the highest transformation yields for both CF and 1,1,1 TCA. Propane-utilizers were much more effective in transforming CAHs mixtures than methane-utilizers. The presence of CF and 1,1,1 TCA in the groundwater had a greater negative effect on ability of methane-utilizers to transform TCE. Methane and propane-utilizers remained activity toward TCE transformation after one year of exposure to increasing TCE concentrations and the transformation of CAH mixtures. The results indicate long-term cometabolic activity can be maintained under microcosm conditions when cometabolism occurs in the presence of ample growth substrate. The batch microcosm methods tested appear to be a reliable method for evaluating the in situ cometabolic bioremediation potential of TCE and CAH mixtures.

Studies with mixed cometabolic substrates evaluated methane-propane and propane-phenol as mixed cometabolic substrates to transform both 1,1,1-TCA and TCE. The mixed substrate studies were performed with enrichments obtained from the McClellan subsurface. No benefit was found in using methane and propane as mixed substrates. More effective transformation was achieved by adding methane alone compared to mixtures of propane and methane.

Propane and phenol as mixed cometabolic substrates showed more promise. The propane and phenol studies were performed in groundwater microcosms and evaluated the transformation of mixtures of 1,1,1-TCA and TCE. The microcosms fed phenol only transformed TCE. The propane culture showed good ability to transform 1,1,1-TCA and some ability to transform TCE. When the microcosms were alternately pulse fed phenol and propane TCE and 1,1,1-TCA was transformed when phenol was fed, and more effective TCE transformation when propane was fed. The ability to transform 1,1,1-TCA when phenol was fed was likely caused by propane-utilizers, since the transformation of 1,1,1-TCA was blocked with high concentrations of propane. Phenol transformation products were also observed when phenol induced 1,1,1-TCA transformation. Propane-utilizers were likely competing with phenol-utilizers for phenol, and cometabolized phenol to form these products.

The propane-utilizers may have been able to obtain energy from these products to drive the cometabolic process. The timing of the pulsed additions was also important. If after propane addition, both 1,1,1-TCA and TCE were added until transformation stopped, and then phenol was added, mainly TCE was transformed. Under these condition phenol-utilizers likely out competed propane utilizers for phenol. However, if phenol was added while propane-utilizers showed some TCE and 1,1,1-TCA transformation activities remaining, phenol transformation products were formed and both 1,1,1-TCA and TCE were transformed. In this case the propane-utilizers likely out competed the phenol-utilizers for phenol, and both TCE and 1,1,1-TCA were transformed. Different microbial communities were likely stimulated by the alternate pulsed addition strategy. The systems fed only phenol eventually lost their TCE transformation ability, while those pulse fed propane and methane remained active towards TCE transformation, and even transformed 1,1,1-TCA. These results suggest a more robust cometabolic system might be achieved through mixed substrate addition of propane/phenol and possibly propane/toluene. The results also show promise in the transformation of CAH mixtures using these mixed cometabolic substrates.

Preliminary studies were also performed on the role of nutrients, with a focus on nitrogen needs. Initial stimulation of the McClellan microcosms indicated that nitrogen was limiting in the groundwater, and fixed nitrogen addition (as nitrate) was required to achieve effective microbial stimulation and transformation of TCE. In studies with McClellan enrichments, however, the most effective transform of TCE by methane-utilizing cultures resulted with prolonged stimulation in the absence of nutrient addition. Transformation yields of 0.20 mg of TCE/mg methane were achieved in microcosms that became nitrogen limited. Enhanced TCE transformation, however, was not observed in propane enrichments that became nitrogen limited. Enrichments were obtained from these microcosms that grew most rapidly in media lacking fixed nitrogen. Effective growth was observed in the

enrichments grown with groundwater, that lacked nitrogen, but had trace nutrients added. Future work is needed to demonstrate that under the nitrogen limited conditions effective TCE transformation was obtained with methane-utilizing microorganisms.

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SECTION I

INTRODUCTION

A. TCE SUBSURFACE CONTAMINATION

Trichloroethylene (TCE) has been widely used as a degreasing agent, popular dry cleaning solvent, and extraction agent (Love and Eilers, 1982; Westrick et. al., 1984) in industries and government facilities including military installations since the 1940s. During 1940 to the 1970s, TCE was also used as an anesthetic by health professionals and extensively used in food production (Frank, 1990). There was no federal, state, or local laws or regulations banning the use of TCE in early 1970s. In mid 1970s, analytical methods became available to measure this compound at low concentrations the soon after TCE was detected in groundwater (Westwick et al., 1994). In the late 1970s, TCE was determined to be a suspected carcinogen (Infante and Tsongas, 1982), and the U.S. Environmental Protection Agency listed TCE as a priority pollutant in 1986, and proposed a maximum contaminant level in drinking water of 5 ppb (U.S. EPA, 1993).

Long-term land disposal of TCE through 1970s occurred at many military installations including McClellan Air Force Base, Sacramento, California. The McClellan AFB, which is the focus of part of this study, is a military installation that has been used to repair aircraft. TCE was used at the site as a degreasing agent and extraction agent. The disposal of TCE in waste pits resulted in contamination of vadose zone and saturated zone. A large TCE contamination source was suspected to be located in the north portion of site 22, beneath the location of waste pits.

Remediation methods, such as pump-and treat, have been used for remediating groundwater contaminated with chlorinated compounds, including TCE (Symon, 1981). However, with a TCE drinking water standard of 5 ppb, pump-and treat remediation is often an inefficient and expensive method for removing CAH pollutants from groundwater. The pumping of groundwater might also result in the transfer of the contaminants to the surface environment. A large volume of contaminated groundwater must also be extracted. The application and operation of pump-and treat is therefore expensive and time consuming. It may require a time scale of decades to clean up a TCE contaminated aquifer to the drinking water standard (Mackay and Cherry, 1989).

Many previous researchers have indicated that in situ bioremediation has good potential to clean up contaminants without bringing groundwater to the surface. This technology may be capable to minimizing remediation costs and may reduce the time required for restoring contaminated aquifers. The contaminants are also completely degraded, and the subsurface can be used as bioreactor to eliminate above ground treatment (Semprini et al., 1991). Field experiments have demonstrated TCE cometabolism under aerobic (Semprini et. al., 1990; Hopkins et al., 1993) and under anaerobic conditions (Beeman et. al., 1994). Aerobic cometabolism and anaerobic reduction now are considered important processes for the bioremediation of TCE and other chlorinated solvents.

B. 1,1,1-TCA SUBSURFACE CONTAMINATION

1,1,1-trichloroethane (1,1,1-TCA) is a volatile organic compound and a common groundwater contaminant in the United States. 1,1,1-TCA is used as a degreasing agent, dry cleaning agent and solvent in various industries. It also can be found in household products such as spot cleaner, glues and aerosol sprays. In 1987 about 700 million pounds of 1,1,1-TCA were produced. 1,1,1-TCA was listed as a volatile organic contaminant under the Safe Drinking Act amendments of the United States (Federal Register, 1989). In 1990, the Environmental Protection Agency had identified more than 200 sites on National Priorities List, that were contaminated with 1,1,1-TCA (ATSDR, 1990).

The widely spread contamination of 1,1,1-TCA is a health threat due to its toxicity. Groundwater contaminated with 1,1,1-TCA was mostly a result of leaking storage tanks, landfill leachates and improper disposal. The contamination of groundwater is of great concern, since it is an important source of water supply for domestic, agricultural and industrial use in the United States. In some states as high as 90% of water supply comes from groundwater (Bedient et al., 1994). EPA regulates 1,1,1-TCA level to be 0.2 ppm (mg/L) in drinking water and 18 ppm (mg/L) in rivers and streams. 1,1,1-TCA is relatively recalcitrant to degradation in nature. Therefore, it is necessary to find a cost effective removal method.

C. PROPERTIES OF CAHS EVALUATED IN THIS STUDY

The groundwater contaminants studied in this report are summarized in Table 1. Trichloroethylene is a synthetic chlorinated organic compound that is highly volatile and colorless. It is also considered as nonflammable and nonexplosive compound at ambient temperature. TCE is commonly used in the industry as a excellent degreasing solvent and extraction agent because its boiling point provides low heat input and facilitates handling of work following degreasing operations. TCE was widely used in the metal-processing industry because it does not react with steel, copper, zinc, or other metal used in the industry. TCE is slightly soluble to water with a limited solubility of 1,100 mg/L at 77 °F and considered to be a highly volatile compound and favorably partitions in air (Mongomery, 1991).

Table 1 Properties of selected groundwater contaminants.

Contaminant Properties	TCE	1,1,1 TCA	CF
Formula	$CHCl=CCl_2$	CCl_3CH_3	$CHCl_3$
Boiling point (°C)	87.2	74.1	61.7
Aqueous Solubility 20 °C (mg/L)	1100	1500	8000
Specific Density 20 °C	1.464	1.339	1.489
Henry's law constant (H_{pc}), 20 °C ($atm.m^3/mol$)	9.9×10^{-3}	1.5×10^{-2}	3.39×10^{-3}
Henry law constant (H_{cc}), 20 °C (dimensionless)	0.342	0.642	0.109
Log Octanol/Water Partition Coefficient.	2.29-3.30	2.18-2.49	1.90-1.97
U.S. Drinking Water MCL (µg/L)	5	200	100

The physical and chemical properties of contaminants also effect the migration and fate of contaminants in subsurface. Sorption is one of the main processes effecting transport in the groundwater and soil. Sorption of the contaminants can be estimated by the octanol/water partition coefficient (K_{ow}). The moderate octanol/water coefficient for TCE indicates some affinity of TCE to sorb onto soil with high organic content. This will slow the movement (retardation) of TCE in an aquifer.

The solubility and specific density also affect the behavior and migration of the contaminants in groundwater. TCE has a greater specific density than water and can sink under gravity into the saturated zone. Thus, TCE is designated as a Dense Nonaqueous Phase Liquid (DNAPL).

Liquid 1,1,1-TCA is colorless. It has high vapor pressure (13.2 kPa at 20°C) therefore it evaporates quickly and has a sweet and sharp odor. With a solubility limit 1500 mg/L at 20°C, 1,1,1-TCA is considered relatively soluble. The Henry's coefficient of 1,1,1-TCA is 0.015 atm-m^3/mol at 20°C. 1,1,1-TCA has a molecular weight of 133.4. The melting point and boiling points are -30.4 °C and 74.1 °C, respectively (Mackay and Shiu, 1982).

D. TCA TRANSFORMATION PATHWAYS

In nature, 1,1,1-TCA can be abiotically transformed as shown in various studies (Vogel et al., 1987; Wing 1997). 1,1,1-TCA can undergo hydrolysis, which results in acetic acid (HOAc), and an elimination reaction, which forms 1,1-dichloroethylene (1,1-DCE). Both processes can occur simultaneously.

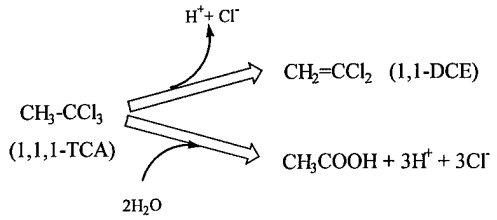


Figure 1 Abiotic transformation pathways of 1,1,1-TCA

1,1,1-TCA transformation rates increase with temperature. The pseudo-first-order rate of 1,1,1-TCA disappearance was reported to be 0.5 to 1.1 years at 25°C and 2 to 3 years at 15°C (Wing, 1997). Acetic acid production is 5 times faster than 1,1-DCE production at 40°C (Vogel et al., 1987). Acetic acid and 1,1-DCE can be transformed under methanogenic conditions.

1,1-DCE → Vinyl Chloride → Ethene
Acetic Acid → CO_2.

1,1,1-TCA can be transformed under anaerobic conditions. Reductive dehalogenation by methanogenic culture converts 1,1,1-TCA to 1,1-dichloroethane (1,1-DCA) by replacing a chloride atom with a hydrogen atom. 1,1-DCA then can undergo further dehalogenation by the same microbes to form chloroethane (CA) (Bouwer and McCarty, 1983; Vogel and McCarty, 1987; Vogel and McCarty, 1987; Galli and McCarty, 1989; Gerkens and Franklin, 1989; Klecka et al., 1990; Doong and Wu, 1996; Wing, 1997). High concentrations of TCA appeared to inhibit the growth of organisms, which was indicated by decrease of cell yield (Galli and McCarty, 1989; Doong and Wu, 1996). The most common substrates used to drive the anaerobic transformations were acetic acid and methanol. 1,1,1-TCA removal increased with substrate addition (Doong and Wu 1996). 1,1-DCA, its anaerobic transformation product, is more recalcitrant.

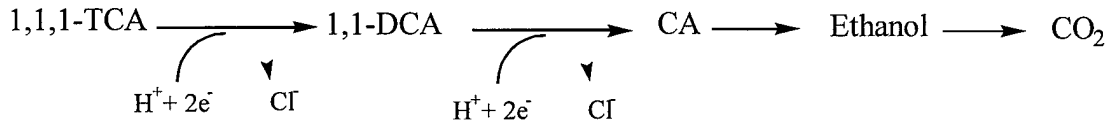
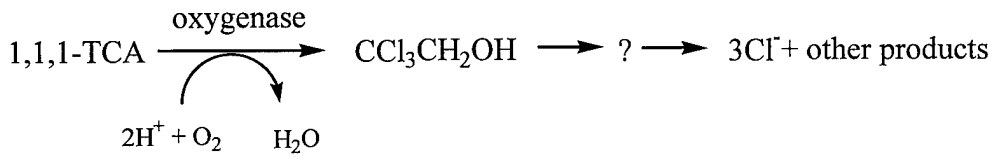


Figure 2 Anaerobic transformation pathway of 1,1,1-TCA

E. AEROBIC TRANSFORMATION OF CAHS

TCE, 1,1,1-TCA, and other CAHs can be aerobically degraded by cometabolism. Cometabolism is considered a metabolism of a xenobiotic compound that does not benefit in growth of the microorganisms (Dalton and Stirling 1982). Wackett (1996) considered co-metabolism of xenobiotic compound to be a function of broad enzyme specificity, imprecise induction specificity, and an evolutionary process that we innately expect to yield biological perfection when that is neither necessary nor, in some cases, thermodynamically feasible. Most CAHs can not support microbial growth, therefore, a growth substrate is needed as an energy source.

TCE, 1,1,1-TCA and other CAHs can be transformed by oxygenase enzymes. There are several types of oxygenase enzymes. Some are non-specific enough to degrade a broad range of CAHs. Only certain microbial populations can produce nonspecific oxygenase enzymes that use oxygen as the electron acceptor and NADH as the reducing energy source to oxidize both growth substrates and non-beneficial substrates. Oxygenase enzymes for example, can transform 1,1,1-TCA by inserting an oxygen atom to form 2,2,2-trichloroethanol.



(McCarty 1993)

Figure 3 Aerobic transformation pathway of 1,1,1-TCA

Since both substrates (beneficial and non-beneficial) have to compete for the same enzyme, the presence of non-beneficial substrate can obstruct the transformation of growth substrates (competitive inhibition) and vice versa. The growth substrates that have been studied extensively can be categorized into two groups; gaseous substrates (methane,

propane and butane) and aromatic substrates (phenol and toluene) (Semprini 1997). Microorganism populations grown on different substrates can have different abilities to cometabolize CAHs, due to the different oxygenase enzymes produced. Some growth substrates such as phenol and non-beneficial substrates such as TCE can be toxic to cultures at high concentration (Chang and Alvarez-Cohen 1995). Transformation products of some chlorinated solvents can also cause inactivation of microbes (Broholm et al., 1990; Alvarez-Cohen and McCarty 1991; Henry and Grbic-Galic 1991; Oldenhuis et al., 1991). Therefore, it is not easy to identify a specific substrate for use as an energy source in cometabolizing CAHs. From the previous works, methane, propane and butane appeared to be potential growth substrates for aerobic cometabolism of 1,1,1-TCA (Semprini, 1997).

F. AEROBIC COMETABOLISM OF TCE

Aerobic microorganisms expressing oxygenase enzymes required for utilizing growth substrates such as methane (Wilson and Wilson, 1985), phenol (Nelson et al.,., 1987), toluene (Nelson et al., 1987; Wackett et al., 1988), ethylene (Henry et al., 1989), ammonia (Arciero et al., 1989), propane (Wackett et al., 1989), propylene (Ensign et al., 1992) have been shown to be responsible for TCE cometabolism.

Inhibitory effects of TCE cometabolism have been observed in previous studies. The transformation of TCE requires expression of oxygenase enzymes and reducing energy source (NADH) to catalyze TCE oxidation. The depletion of enzymes and NADH significantly reduces TCE transformation ability (Chang and Alvarez-Cohen, 1995). In addition, the competition of TCE for the active sites with substrate results in decreases in the TCE transformation rates. Direct toxicity of TCE at high concentrations and TCE transformation product toxicity also inhibits TCE transformation (Alvarez-Cohen and McCarty, 1991; Oldenhuis et al., 1991).

1. TCE oxidation by methanotrophic bacteria (methane-utilizers)

Methane-utilizing bacteria have been extensively studied for 25 years. Methane-utilizing microorganisms are widespread in transition zone between aerobic and anaerobic zones in subsurface where methane and oxygen are present (Hanson, 1980). These microorganisms are commonly called methanotrophs. The pathway for methane degradation shown in Figure 4 was first documented by Dalton and Stirling, 1982. In the first step, the enzyme methane monooxygenase (MMO) oxidizes methane to methanol. The methane oxidation reaction requires $NADH_2$ as an electron donor, which is generated in the last two steps of the pathway. Methanol is continuously transformed to formaldehyde, which is either metabolized into bacteria biomass or further oxidized to formate and carbon dioxide. The oxidation of formaldehyde to formate and the oxidation of formate to carbon dioxide generate $NADH_2$, required for the initial oxidation of methane.

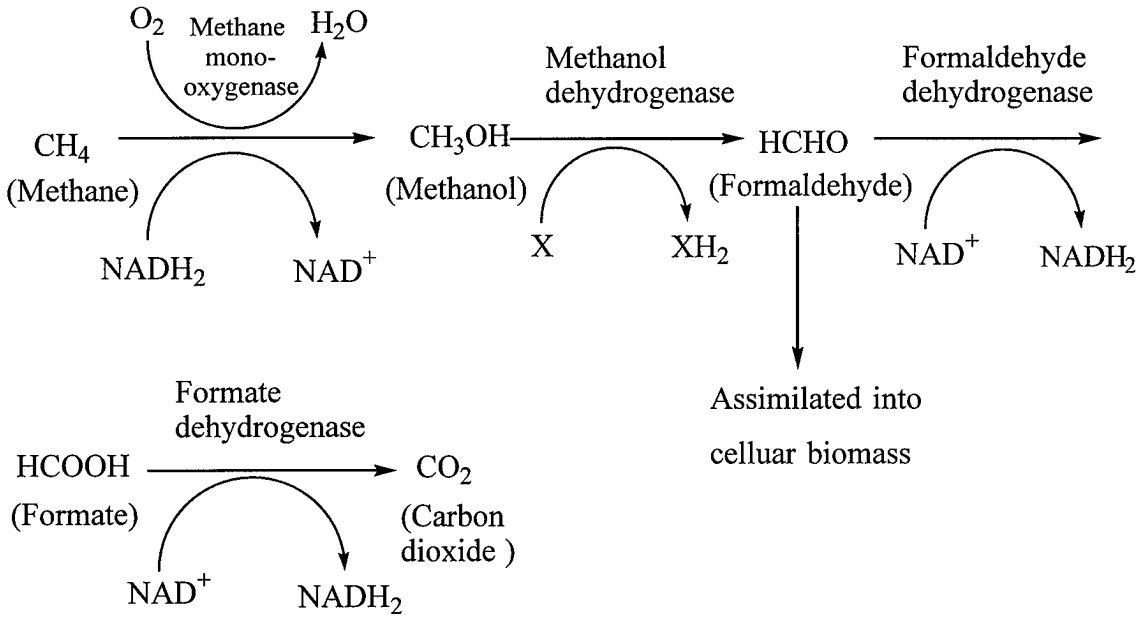


Figure 4 The pathway for methane oxidation by methanotrophic bacteria (Dalton and Stirling, 1982).

Methane-utilizing bacteria that cometabolized TCE was first discovered by Wilson and Wilson (1985). Their observations suggested that the enzymes that epoxidate ethylene transform TCE. After Wilson 's discovery, extensive research on TCE cometabolism by methanotrophic microorganisms was conducted (Fogel et al., 1986; Strand and Shippert, 1986; Hanson et al., 1980). Fogel et al., 1986 showed that five chlorinated compounds including trichloroethylene (TCE), vinyl chloride, and cis- and trans-1,2-dichloroethylene, but not tetrachloroethylene, could be oxidized by methane-utilizing bacteria. The study also speculated that TCE epoxide might be a product of TCE oxidation by MMO. The study indicated that MMO is highly nonspecific enzyme, because most methanotrophs are capable of utilizing methane and other C_1 compounds as sole sources of carbon and energy.

The pathways for aerobic cometabolic degradation of TCE were investigated by Little et al., (1988) (Figure 5). TCE was oxidized by MMO to TCE epoxide or trichloroacetaldehyde, which was hydrolyzed spontaneously to form dichloroacetic acid, glyoxylic acid, or one-carbon compounds such as carbon monoxide and formate (Little et al., 1988; Oldenhuis et al., 1990; Fox et al., 1990). The trichloroacetaldehyde is oxidized to trichloroacetate and partially transformed to trichloroethanol (Newman et al., 1991). Some carbon from TCE is incorporated into cells and converted to CO_2 (Fogel et al., 1986; Little et al., 1988).

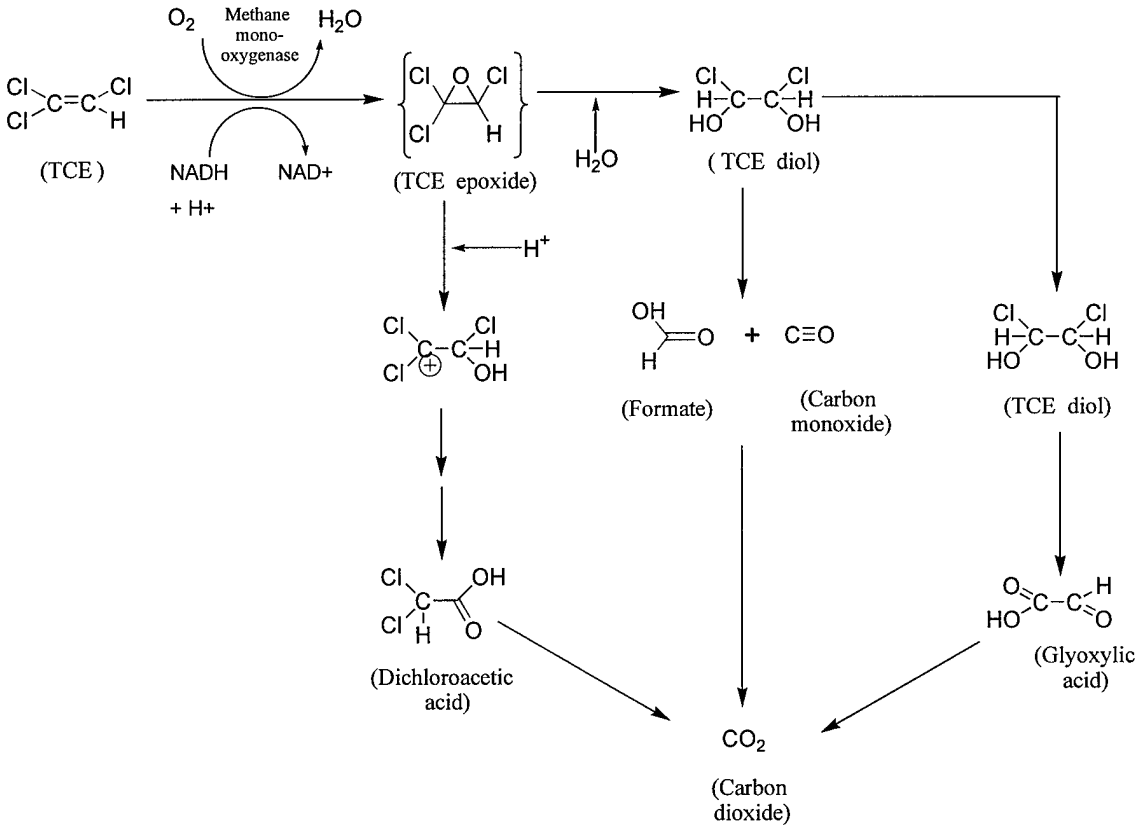


Figure 5 The mechanism of TCE transformation by methanotrophic bacteria (Little et al., 1988).

TCE transformation by-products have been found to inhibit TCE degradation (Alvarez-Cohen and McCarty, 1991; Oldenhuis et al., 1991; Henry and Grbic-Galic, 1991). However, hydrolysis products of the TCE epoxide and trichloroacetaldehyde did not inactivate MMO (Fox et al., 1990). Other resting cell studies showed that the depletion of electron donor for NADH regeneration and the product toxicity of TCE cometabolism are the factors affecting of the TCE transformation rate and transformation capacity. Methane, oxygen and TCE utilization were greatly reduced after TCE transformation occurred. Similar observations were documented in studies of chloroform (CF) and TCE degradation by methane-utilizing bacteria (Alvarez-Cohen and McCarty, 1991). They speculated that phosgene and TCE epoxide were responsible for the observed product toxicity of CF and TCE, respectively. Studies on competitive inhibition also revealed that high methane concentrations have negative effect on TCE transformation (Broholm et al., 1992; Oldenhuis et al., 1991; Semprini et al., 1991). High methane concentrations significantly compete with TCE for active site on MMO enzyme, resulting in a reduction of TCE transformation rates.

Methanotrophic biostimulation field experiments and laboratory column studies have demonstrated that the cometabolic biostransformation of chlorinated alkenes resulted from

the biostimulation of indigenous methanotrophic bacteria (Semprini et. al., 1990; Broholm et al., 1991). The studies of in situ bioremediation indicated that degradation of TCE and others CAHs in soil and groundwater is complex and developing effective models for TCE and CAHs degradation is difficult. However, model simulations of in situ bioremediation of CAHs at Moffett Field site, including competitive inhibition between chlorinated ethylene and methane, successfully fitted the field experimental data (Semprini and McCarty, 1992).

Mixed culture and pure cultures of methanotrophic bacteria have been studied to enhance cometabolic degradation of TCE (Broholm et al., 1993; Oldenhuis et al., 1989 and 1991). Methanotrophs isolated from a TCE-contaminated aquifer, type II *Methylosinus Trichosporium* OB3b, expressing soluble methane monooxygenase, oxidized TCE at high rate under copper limited growth conditions. Long term TCE transformation activity was observed. The highest transformation rate of TCE (200 nmole min^{-1} mg of $cell^{-1}$) by the OB3b culture was documented by Oldenhuis et al (1991). The rate of TCE transformation was comparable to the rate of methane degradation.

In addition to TCE, *M. Trichosporium* OB3b also degraded dichloromethane, chloroform, dichloroethane, cis- and trans-DCE, and 1,2 dichloropropane. This pure culture could not oxidized carbon tetrachloride and perchloroethylene (Oldenhuis et al., 1989 and 1991). Similar observations were achieved in batch and chemostat reactor studies with mixed methanotrophs and pure (*M. Trichlosporium* OB3b) methanotrophs (Chang and Alvarez-Cohen, 1996). Resting cell studies indicated that CAH product toxicity to cometabolic degradation by these cultures decreased with increasing chlorine substitution.

Kho et al (1993) discovered a soluble methane monooxygenase produced in Type I methanotrophs, *Methylomonas Methanica* strain 68-1. TCE degradation by whole-cell sMMO activity of 68-1 was comparatively higher than sMMO activity in *Methylosinus Trichosporium* OB3b grown under the same conditions when copper was present. The research also showed that MMO gene probes from OB3b are almost genetic homology to those found on 68-1.

Broholm et al (1991; 1993) studied the different abilities of eight mixed culture of methane-oxidizing bacteria to degrade TCE. The experiment was conducted in batch reactors, at 10 °C, a common temperature for soils and groundwaters. TCE degradation was observed on three of the eight mixed cultures, when the cultures were grown on methane. These three cultures were also able to transform TCE during the oxidation of methanol. These experiments demonstrated the ability of mixed cultures to degrade TCE varied significantly, even though all cultures were grown under the same conditions. The study also included model simulations for TCE degradation and methane oxidation. The model based on competitive inhibition kinetics was applied to laboratory batch experiments. The proposed mathematical model describing the growth of bacteria and the transformation of TCE and uptake of methane successfully simulated the experimental results.

Chang and Alvarez-Cohen (1995) compared methane, propane, toluene, and phenol-utilizers ability to transform TCE with microbes enriched from a contaminated site. All cultures were grown under chemostat conditions. The transformation capacity (Tc), which represents as the mass of TCE transformed divided by resting cell mass, was determined. The methane culture exhibited the highest transformation capacities for TCE, CF, and 1,2-DCA. The TCE transformation capacities (Tc : mg TCE / mg cells) of the four cultures were as follows: methane, 0.05; phenol, 0.031; toluene, 0.0073; and propane, 0.0065. The transformation yields (Ty : mg TCE / mg growth substrate), which represents the mass of CAH degraded per mass of growth substrate utilized, were; methane, 0.017; propane, 0.0056; toluene, 0.0021; and phenol, 0.017. The propane and methane cultures were able to transform TCE, 1,2-DCA and chloroform. The phenol and toluene transformed only TCE. None of cultures tested were able to transform PCE and CCl_4.

Transfomation kinetics of chlorinated ethenes, including TCE, by *Methylosinus Trichosporium* OB3b were reported by Van Hylckama Vlieg et al (1996). The detection of unstable epoxides of chlorinated ethenes was observed by using on-line gas chromatography analysis. The kinetics of all chlorinated alkene transformation and the corresponding epoxides by *Methylosinus Trichosporium* OB3b expressing pMMO or sMMO were detected. The study found significant amounts of all the epoxides, except 1,1-DCE epoxide, leaving the cell. The results of the study also showed that methane and acetylene inhibited the degradation of cis 1,2-DCE epoxide, suggesting that cis 1,2-DCE epoxide is transformed by sMMO.

2. TCE oxidation by propane-utilizing microorganisms

Previous work has documented that microorganisms are able to use propane as a growth substrate under aerobic conditions. Propane-oxidizers have been enriched from soil and water samples (Perry, 1979; Hou et al., 1983). These microorganisms are able to degrade a broad range of aliphatic hydrocarbons. The propane oxygenase enzyme is also nonspecific enough to metabolize and oxidize short-chain alkenes (Hou et al., 1983) and other aliphatic hydrocarbons. The first oxidation step is to insert O from O_2 into propane molecule to form 2-propanol, which is further oxidized to acetone (Perry, 1980). However, other studies by Stephen and Dalton (1986) concluded that the initial propane oxidation takes place on the terminal carbon atom in propane molecule.

In contrast with the significant study of methane-utilizing bacteria, little work have been done on study CAH cometabolism by propane-utilizing bacteria. To date, no work has studied the application of these microorganisms for in-situ bioremediation. Wackett et al., (1989) first demonstrated that propane monooxygenase (PMO) could catalyze the oxidation of TCE. The propane monoxygenase enzyme from five different bacteria could oxidize TCE when propane was used as a growth substrate. Inhibition between propane and TCE was observed on this study, indicating that propane monooxygenase enzyme was involved in TCE degradation. In addition to TCE, propane monooxygenase in *Mycobacterium Vaccae* JOB5 transformed vinyl chloride and cis-and trans-DCE, but not tetrachloroethylene.

The degradation and inhibition of TCE and 1,1,1 TCA has been studied with a propane-oxidizing enrichment culture (Keenan et al., 1993). This is the first work demonstrated the degradation kinetics and inhibition of TCE and 1,1,1 TCA cometabolism by propane. The results of the study showed that propane inhibited TCE degradation and the kinetics were best described by noncompetitive inhibition model. TCE degradation followed Michaelis-Menten kinetics with a V_{max} = 0.0016 mg TCE / mg TSS-hr and a K_s = 0.6 mg TCE/L. The results also demonstrated that TCA was strongly inhibited by propane and a competitive inhibition model did not fit the experimental data.

G. COMETABOLISM OF 1,1,1-TCA

Methane-oxidizing bacteria displayed an ability to cometabolize 1,1,1-TCA (Broholm et al., 1990; Strand et al., 1990). These bacteria express methane monooxygenase (MMO), which inserts an oxygen atom into a methane molecule and initiates the production of methanol. MMO is also responsible for transforming solvents in a similar manner. An oxygen atom is inserted into the 1,1,1-TCA molecule creating an OH group, producing 2,2,2-trichloroethanol (CCl_3CH_2OH). Since MMO is responsible for transforming both growth substrates, substrate competitive inhibition occurs. Results from studies apparently confirm the existence of competitive inhibition (Broholm et al., 1990; Strand et al., 1990).

Strand et al (1990) showed that methane degradation followed Monod kinetics while TCA transformation followed first order kinetics with rate constants of 8.8×10^{-5} L/mg VSS-h, for concentrations less than 3000 μg/L. 1,1,1-TCA transformation was inhibited by methane at concentrations over 0.25 mg/L. However, after methane was depleted, 1,1,1-TCA transformation continued for 65 hrs. Broholm et al (1990) reported a decrease in methane consumption rate with an increase of 1,1,1-TCA concentrations. TCA degradation rate decreased with the increase of methane concentration.

1. Cometabolism of 1,1,1-TCA by butane-utilizers

Kim et al (1997) reported microorganisms grown on butane from the Hanford DOE site had abilities to degrade a broad range of CAHs including 1,1,1-TCA, 1,1-DCE and its abiotic transformation products. Soil microcosm studies showed that aqueous concentrations of 1,1,1-TCA as high as 2400 μg/L could be transformed completely. They observed about 70% dechlorination of 1,1,1-TCA that was transformed. Although 1,1,1-TCA transformation was slower than 1,1-DCE and other chlorinated ethanes, it is still considered to have a fair transformation potential. Resting cell experiments showed that 1,1,1-TCA did not cause significant cell inactivation after the cell was exposed to 1,1,1-TCA. The information on butane-utilizers is limited. Although there were reports of various strains of bacteria and molds grown on butane (Davis 1964; McLee et al., 1972; Phillips and Perry 1974; Van-Ginkel et al., 1987), no study on their abilities to cometabolize CAHs was performed in these studies. The butane-utilizers isolated could grow on other substrates such as propane, ethane, iso-butane, pentane and hexane, but not on methane (McLee et al., 1972; Van-Ginkel et al., 1987).

McLee et al (1972) compared the readiness for growth among n-butane, n-butanol, which is the first metabolite of n-butane oxidation, and glucose, which is a more highly oxidized substrate. Isolates grown on n-butane showed slower growth than those grown on n-butanol and glucose. The similarity of growth rate on n-butanol and glucose indicated that the degree of reduction did not affect the utilization of the growth substrate. Further studies concluded that oxygen partial pressure or butane partial pressure did not affect butane degradation rate. *Nocardia* TB1, which could grow in butane and several straight-chain hydrocarbons, but not on 1-alkenes was likely to contain an alkane-type monooxygenase with broad substrate specificity (Van-Ginkel et al.,. 1987). This non-specific enzyme was responsible for butane oxidation and might be responsible for 1,1,1-TCA oxidation mentioned earlier. The butane transformation pathway is shown Figure 4. There are no extensive studies on the oxygenase enzyme system in butane-utilizers. It is possible that butane, similar to methane-utilizers, can have more than one type of oxygenase enzyme, and not all may not be able to degrade 1,1,1-TCA.

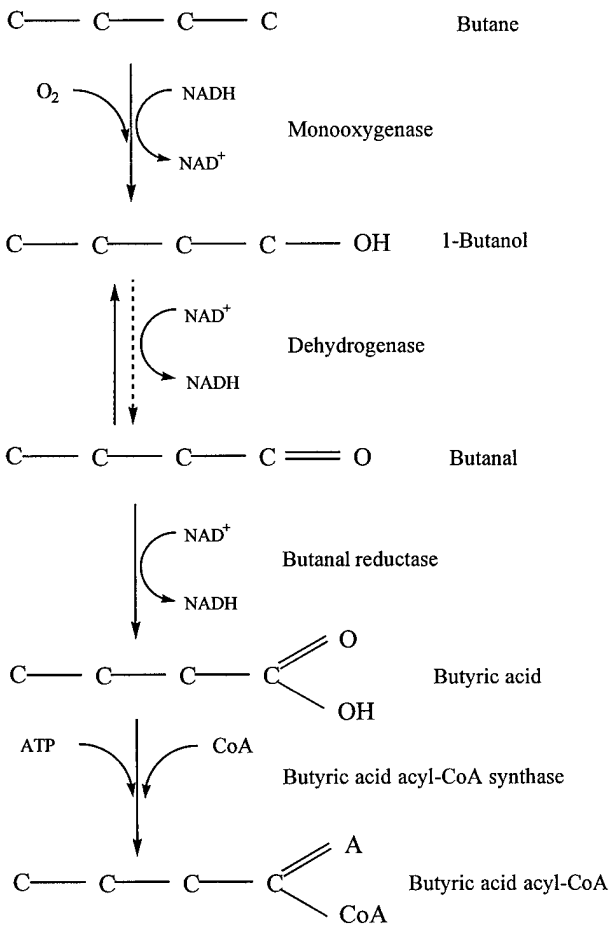


Figure 6 Butane transformation pathway (the hydrogen bonds to the carbon are omitted.)

2. Cometabolism of 1,1,1-TCA by propane-utilizers

Several strains for propane-utilizers isolated from soil and water samples were able to use propane as a sole energy and carbon source. A partial list includes; Mycobacterium (Perry 1980; Hou et al., 1983), Acinetobacter, Actinomyces, Brevibacter, Norcardia, Pseudomonas (Hou et al., 1983), Arthrobacter (Stephens and Dalton, 1986), Rhodococcus (Murrell and Woods, 1989). Previous studies showed that several of these propane-utilizers have an ability to degrade various CAHs. *Mycobacterium vaccae* JOB5 transformed trichloroethylene (TCE), 1,1-dichloroethylene (1,1-DCE), trans-1,2-dichloroethylene (trans-1,2-DCE), cis-1,2-dichloroethylene (cis-1,2-DCE) and vinyl chloride (VC) (Wackett et al., 1989). Studies with propane-utilizers enrichments showed abilities to transform chloroform (CF), TCE and 1,2-dichloroethane (1,2-DCA) (Chang and Alvarez-Cohen, 1995). 1,1,1-TCA cometabolism by propane-utilizers enrichments was first reported in 1993 (Keenan et al., 1993). Kim (1997) reported 1,1,1-TCA transformation by propane-utilizers enriched from the Hanford DOE site. Propane strongly inhibits 1,1,1-TCA transformation (Keenan et al., 1993). Inhibition models including competitive, noncompetitive and uncompetitive inhibition did not fit the data (Keenan et al., 1993).

Propane-utilizers express an oxygenase enzyme, which was identified as propane monooxygenase enzyme (PMO) (Wackett et al., 1989). Various studies showed that PMO is a non-specific enzyme with a broad substrate range and able to oxidize short-chain alkenes and other aliphatic hydrocarbons (Hou et al., 1983; Wackett et al., 1989). Apparently, PMO is responsible for 1,1,1-TCA transformation. Propane is oxidized by inserting an oxygen molecule, converting propane to 2-propanol, which is further oxidized to acetone (Perry, 1980). However, Stephens and Dalton (1986) concluded that propane can also undergo terminal oxidation pathway and be converted to 1-propanol. This indicates that PMO is non-specific with respect to the site of oxygen atom insertion (Stephens and Dalton, 1986).

H. CHLORINATED ALIPHATIC HYDROCARBONS AT MOFFET FIELD

Moffett Federal Airfield (formerly the Moffett Naval Air Station), Mountain View, CA, was contaminated with various chlorinated aliphatic hydrocarbons (CAHs) including 1,1,1-TCA. Since 1985, there have been several studies on transforming CAHs at the site by aerobic cometabolism. Phenol, toluene, methane, ammonia and propane were tested for their abilities to cometabolize CAHs both in-situ and in microcosms (Hopkins et al., 1993; Semprini et al., 1993; Hopkins and McCarty, 1995). Field studies were done on methane (Semprini et al., 1990; Semprini et al., 1991), phenol (Hopkins et al., 1993; Semprini et al., 1993; Hopkins and McCarty, 1995) and toluene (Hopkins and McCarty, 1995). Microcosm studies were performed with phenol (Hopkins et al., 1993; Semprini et al., 1993), methane (Lazarone and McCarty, 1990; Semprini et al., 1993), propane (Lazarone and McCarty, 1990), toluene and ammonia (Hopkins et al., 1993). The studies verified that groundwater microorganisms can be stimulated by adding each primary substrate to groundwater in the presence of oxygen.

Phenol and toluene utilizers were easier to stimulate than methane and ammonia-utilizers. Phenol and toluene-fed microcosms degraded TCE and cis-DCE effectively. Methane-fed microcosms were effective in degrading trans-DCE and some TCE. The ammonia fed microcosm was the least effective in degrading all CAHs. Propane-fed microcosms showed no propane utilization. No substrate was effective in 1,1,1-TCA transformation.

I. BIOAUGMENTATION

One potential limitation of bioremediation is the inability of indigenous microorganisms to degrade the contaminant of interest. There are possible several causes such as: a low population, the lack of suitable strains, the lack of appropriate enzymes and an improper environment (Vogel, 1996). In these cases, adding suitable strains into the site can activate the degradation process. This procedure is known as bioaugmentation, which does not only mean addition of outside microorganisms, but also includes the re-inoculation of indigenous microorganisms directly from the same source to amplify a particular biological activity. The process has been used in various fields such as wastewater treatment, soil and groundwater remediation, and agriculture (Vogel, 1996). There have been several bioaugmentation microcosms studies for groundwater remediation (Munakata-Marr et al., 1996; Munakata-Marr et al., 1997). Bioaugmentation allows inoculation of strains proven to degrade target contaminants to the site. One of the major concerns is creating and maintaining the appropriate condition for the inoculate strain to survive and grow. Bioaugmentation is not a new technology. It was first practiced in 1970 for cleaning up an ocean oil spill site, *Torrey Canyon*, in United Kingdom (Frosyth et al., 1995).

There are several parameters that need to be considered in bioaugmentation: pollutant characteristics, (e.g. bioavailability, concentration, microbial toxicity) and soil physico-chemical characteristics (humidity, organic matter content, clay content and pH), microbiology (e.g. enzyme stability and activity) and methodology (e.g. method of inoculation) (Vogel, 1996).

Munakata-Marr et al (1996) bioaugmented small-column microcosms with *Burkholderia (Pseudomonas) capacia* G4 (G4) and $PR1_{301}$, which are phenol-utilizers, and compared their performance with indigenous phenol-utilizers stimulated from Moffett aquifer material and groundwater. G4 expresses toluene ortho-monooxygenase enzyme (TOM), which can be induced by phenol or toluene and is capable of transforming TCE. $PR1_{301}$ is a nonrecombinant NTG-induced mutant of G4 and is capable of constitutively expressing TOM while grown on substates such as lactate. The lactate-fed microcosms performed poorer than the phenol-fed bioaugmented microcosms. But in semicontinuous bioaugmentation microcosms, lactate was shown to enhance TCE degradation in phenol-grown resting mixed cultures. Microcosms sterilized prior to bioaugmentation and then inoculated with G4 and $PR1_{301}$ had higher transformation yield than microcosms with indigenous microorganisms. This study showed that by adding selected strains of phenol-utilizers, immediate activity could be expected, therefore, eliminating long lag periods. When the selected strain is augmented into non-sterile conditions, the major concern is the

survival of introduced organisms. The added organism may fail to compete with native strains or may be preyed on (Munakata-Marr, 1996).

A bench-scale study and a filed experiment of inoculating *Pseudomonas stuzeri* strain KC for remediating carbon tetrachloride (CT) contaminated groundwater site at Schoolcraft, Michigan, demonstrated that colonization of strain KC could be achieved by modifying the alkalinity (Mayotte et al., 1996; Dybas et al., 1997). Acetate, phosphorus and base were added to the column microcosms' exchange fluid to create favorable conditions for growth of strain KC. The study confirmed that bioaugmentation of strain KC is feasible with the right niche adjustment. CT transformation was immediate and up to 70% transformation efficiency was observed (Mayotte et al., 1996). A field experiment achieved 50-80% CT transformation efficiency with the strain KC augmented and colonized. The pH of the subsurface environment was adjusted to be slightly alkaline to accommodate the strain KC (Dybas et al., 1997).

Toccalino et al (1993) studied the effects of nitrogen on propane and butane biodegradation in an unsaturated sandy soil. The results indicated that butane-utilizing bacteria overcame nitrogen limitations. Nitrogen fixation was not observed on propane-amended soil. Kim et al (1997) demonstrated butane-utilizing microorganisms from the subsurface of Hanford DOE site, WA capable of transforming CF and 1,1,1-TCA in microcosms.. Effective CF transformation was induced by butane-utilizers, with complete transformation of 1200 ug/L of CF in aqueous solution. The study also concluded that oxygenase enzymes of butane utilizers are involved in CF transformation. This is the first demonstration of butane as a cometabolic substrate for CAH transformation.

Hanamura et al (1997) evaluated chloroform, TCE and other CAHs with pure cultures of butane-utilizing bacteria and an enrichment obtained from the microcosms of Kim et al., (1997). CF transformation was observed on butane grown *Pseudomonas butanovora* and Myco bacterium vaccae JOB5, and the enrichment CF8. Using enzyme- blocking tests they demonstrate the same monooxygenase enzyme was responsible for CF transformation and butane oxidation. CF8 and *P. butanovora* were able to degrade other chlorinated hydrocarbons including TCE, 1,2-cis-dichloroethylene, and vinyl chloride.

J. THE EFFECT OF NUTRIENTS ON AEROBIC COMETABOLISM

Meeting nutrient requirements to maintain effective microbial growth in the subsurface environment is one of the major factors that influence CAH cometabolism potential for in situ bioremediation. Nitrogen, particularly nitrate, is one of the most essential nutrients that is often found to be limiting in subsurface aquifers. The addition of nitrogen sources such as nitrate or ammonia to the nitrogen-deficient subsurface may enhance TCE and CAHs degradation.

Methane-utilizers are categorized into two groups (Type I and II) based on their internal membranes. Both types can express particulate MMO (pMMO). Type II methanotrophs can

express sMMO (soluble forms) that are responsible to transform a broad range of substrates, and are most active toward TCE transformation. sMMO can be produced under the copper limited conditions. In contrast, Type I organisms that express pMMO require copper for growth (Brusseau et al., 1990; Oldenhuis et al., 1989; Tsien et al., 1989).

Type II methanotrophs appear to be selected during nitrogen-limited conditions. Type I strains appear to be present in almost all methane-enrichment locations when nutrients such as nitrogen and copper are available. Graham et al. (1993) showed that *M. Trichosporium* OB3b, Type II strains, can be selected under nitrogen limitations. Type I organisms were unable to fix molecular nitrogen, while Type II methanotroph can typically fix nitrogen and expressing sMMO at low oxygen tensions. Nitrogen-fixing methane-oxidizers, grown at low oxygen tension, were also found to degrade TCE rapidly and exhibited high TCE transformation capacity (Chu and Alvarez-Cohen, 1996). These results suggest that reactor systems can be manipulated to select for methane utilizing bacteria with high TCE transformation ability. On the other hand, most selection under in-situ conditions is less promising due to copper availability in the subsurface (Graham et al., 1993).

Methane-utilizers can produce poly-β-hydroxybutyrate (PHB) as an endogenous energy source under nitrate limited conditions for regeneration of NADH during TCE transformation (Asenjo, J. A. et al., 1986; Henrysson and McCarty, 1993). PHB is an intracellular reserve polyester polymer whose synthesis serves as an electron sink for microorganisms under limited growth conditions such N, P, S, Mg , and/or O_2 limitations (Dawes and Senior, 1973). The intracellular reducing equivalents to improve and extend TCE transformation might be due to the catabolism of stored PHB content in methane utilizers (Henrysson and McCarty, 1993; Henry and Grbic-Galic, 1991). High accumulation of PHB content was also observed upon depletion of with *Methylosinus Trichosporium* OB3b (Shan et al., 1996).

The effect of the nitrogen on propane and butane-utilizers was also evaluated in an unsaturated sandy soil (Toccalino et al., 1993). Microorganisms in the soil amended with nitrate degraded butane and propane more rapidly than nitrogen limited controls. However, the butane-amended soil overcame their nitrogen limitation by fixing nitrogen.

Compared with methanotrophic bacteria cellular-lipids studies, no work has documented the influence of endogenous storage lipids (PHB) during cometabolism of TCE by propane and butane-utilizers. In addition, no work has shown the effect of nutrient addition on TCE transformation by butane and propane microorganisms. The synthesis of cellular lipids on these organisms and the effect of nutrient have also not yet clearly identified. One study has documented the large accumulation of cellular lipids of a Nocardia strain grown on propane and butane (Davis, 1964). There are at least three lipid products which accumulate to the Nocardia cells such as glyceride, aliphatic waxes and structures similar to poly-β-hydroxybutyrate. All of these materials may be considered as carbon and energy reserve materials.

Nutrient levels can be a critical issue in bioaugmentation. It is necessary for maintaining the efficiency of inoculated strains in bioaugmentation. Laboratory and field experiments showed that adding nitrogen and phosphorus can enhance microbial activity of methanotrophs and TCE transformation (Brockman and Payn, 1995; Palumbo et al., 1995). Most laboratory enrichment cultures isolated are grown with excessive nutrient supplements to assure the maximum growth is achieved. When these cultures are inoculated into subsurface conditions, where nutrients are less abundant, poorer performance can be expected. Nutrient conditions may need to be adjusted to assure the survival and effectiveness of inoculated microorganisms. Most effective bioaugmentation might be achieved using strains that grow effectively under the nutrient conditions of the subsurface. Methods are needed to track the bioaugmented cultures in the subsurface. A PCR method was applied in our research in an attempt to determine if bioaugmented enrichments differ from the indigenous strains.

K. PCR METHODS

"Polymerase Chain Reaction (PCR) is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA" (Taylor, 1991). PCR can multiply DNA molecules by up to a billionfold in a test tube, yielding large amounts of specific genes for cloning, sequencing or mutagenesis purposes (Madigan et al., 1997). PCR eliminates culturing microorganisms, thereby allowing increased sensitivity in detecting DNA sequences present in small amounts in samples with DNA from mixed populations (Atlas and Steffan, 1988).

PCR has been widely used in the areas of molecular biology, diagnostics, systematics and forensic science, due to its high specificity, sensitivity and consistency (Bej and Mahbubani, 1994). In environmental microbiology, PCR has been used for identifying microorganism population, monitoring shifting in microbial population and monitoring microbial survival in bioaugmentation (Burlage, 1995; Vogel, 1996 ; Tiedje, 1997; Petrich, 1995 ; Bej, 1994 ; (Atlas and Steffan, 1988). PCR allows the study of bacterial diversity in complex environments, where only a small percentage of the indigenous microorganisms can be isolated (Picard et al., 1992).

One of the important issues in applying PCR with environmental samples is the preparation of samples to recover contaminant-free nucleic acids. Since most environmental samples contain humic materials, clay and other organics that can potentially inhibit the PCR reactions, extraction and purification of nucleic acid becomes critical (Picard et al., 1992; Bej and Mahbubani, 1994). PCR involves three stages (Steffan and Atlas 1991): 1) DNA is melted to convert double-stranded DNA to single-stranded DNA; 2) Oligomer primers are annealed to the target DNA; and 3) The DNA is extended by nucleotide addition from the primers by the action of DNA polymerase. The primers are designed to hydridize to regions of DNA flanking a desired target gene sequence. The primers are then extended across the target sequence using DNA polymerase (or Taq DNA polymerase) in the presence of free deoxynucleotide triphosphates, resulting in a duplication of the starting target

material. Melting the product DNA duplexes and repeating the process many times results in an exponential increase in the amount of target DNA. Amplified DNA can then be separated and identified by electrophoresis in agrose gel or polyacrylamide gel (Caetano-Anolles and Bassam, 1993). The differences or similarities of each sample can be identified by the bands' location and intensity.

L. REACTOR SYSTEMS FOR THE BIOREMEDIATION OF TCE AND OTHER CAHS

Experimental bioreactors have been designed to study the cometabolic degradation of TCE and other CAHs. Many types of bioreactors using methanotrophic bacteria have been studied including a biofilm reactor with continuous purging of methane and oxygen (Strand et al., 1990), a two-state bioreactor (a dispersed-growth reactor followed by a plug flow reactor) (Alvarez-Cohen et al.,., 1991; McFarland et al., 1991), a sequential anaerobic-aerobic reactor system for mixed chlorinated solvents treatment (Long et al., 1991), and a multi-state bioreactor with a methane-utilizing pure culture (Tschantz et al., 1995).

Since competitive inhibition greatly affects the transformation of TCE and the utilization of the growth substrate (as methane), several researchers have constructed reactors to avoid competitive inhibition to increase TCE transformation efficiency. The dual or multiple reactors configurations described above have some advantages over a single reactor. Here, the cells are grown in the absence of the CAHs, and mixed with the CAHs in the absence of the growth substrate for CAHs treatment. The cells have a finite capacity to transform CAHs in the absence of the growth substrate. Competitive inhibition between the growth substrate and TCE are avoided because the growth and transformation processes are separated.

There have been several reports of the effect of TCE loading on methanotrophic cultures in the reactors (Strand et al., 1990; Strand et al., 1991). A mixed methanotrophic culture was maintained with continuous supply of methane and nutrients with TCE loading increasing from 4 to 10 μg TCE/ (mg protein-d). The maximum sustainable ratio of TCE transformed to methane consumed was 6 μg TCE/mg methane. The study concluded that aerobic cometabolism of TCE by methanotrophs in a continuous TCE-fed system is an unstable process. The degradation of TCE can not be maintained due to product toxicity. Population shifts and changes in enzyme activity occurred with long-term exposure of a mixed culture to concentrations levels of TCE.

The kinetics of methane utilization and the cometabolic degradation of TCE and 1,1,1 TCA by mixed methanotrophic culture were studied in close-system reactor (Strand et al., 1990). Continuous increases of TCE into the reactor showed that the activity of methanotrophic culture ceased at aqueous TCE concentrations of 7,770 μg/L. However, dissolved TCA concentrations less than 4,470 μg/L had no inhibiting effects on methane oxidation rates. The results also showed that for TCA, but not TCE, biodegradation rates were inhibited by the presence of dissolved methane at concentrations in excess of 0.25

mg/L. Lower TCE and TCA biodegradation rates were observed for mixtures of TCE and TCA.

Besides a bioreactor fed with single methane as growth substrate, there is one report using methane and propane as mixed substrates for a continuous-recycle packed and expanded bed bioreactor (Phelps et al., 1990). This study has shown substantial TCE degradation in a reactor fed both methane and propane. When methane alone was added to the reactors as an sole of energy source, TCE transformation decreased by about 60%, compared with the reactor in which both methane (5% by volume) and propane (3% by volume) were fed. When propane alone was added to the reactor, the extent and rate of TCE degradation was similar to that observed when methane and propane were fed. The increased efficiency of propane mixed with methane, or with propane alone indicated that the consortia use propane more efficiently as a cometabolic substrate, or that propane does not complete as effectively as methane with TCE-transforming enzymes. The results of the study also indicated that propane-fed reactor more effectively transformed TCE than the methane-fed reactor.

SECTION II

INITIAL EVALUATION OF MICROBIAL ACTIVITIES AT THE MCCLELLAN, EDWARDS, AND MOFFETT AFB

A. INTRODUCTION

This work has focused on determining the potential for aerobic cometabolism of CAHs of interest to non-toxic end products using methane, propane and butane as cometabolic growth substrates. The initial phase of this work was to screen different sites for field demonstrations of in-situ cometabolism using these substrates. Based on the results of this initial screening more detailed laboratory investigations were performed for selected sites, with an emphasis on systems likely to be employed in the field demonstrations. The results of these more detailed laboratory investigations are presented in Sections III, IV, and V of the report. The work completed herein is directed toward sites of interest to the Air Force, including, McClellan AFB, Edwards AFB, and Moffett NAS. Subsurface microorganisms grown on methane, propane, and butane were enriched from subsurface materials from these sites and their transformation of CAHs of interest was evaluated.

B. INITIAL EVALUATION OF MICROBIAL ACTIVITIES AT MCCLELLAN AFB

Trichloroethylene (TCE) is one of the most widespread contaminants in soil and groundwater, due to its use as a degreaser, dry cleaning solvent, and extraction agent in industry and government facilities including military installations (Imfante and Tsongas, 1982; Westrick et al., 1984). Long-term land disposal of TCE through 1970s occurred at many military installations including McClellan AFB. TCE concentrations greater than 0.5 mg TCE/L have been detected in groundwater at the McClellan AFB.

1,1,1 TCA and CF are also among the most widespread contaminants in groundwater and soil. Like TCE, both compounds are widely used as industrial solvents and military extraction agents. 1,1,1 TCA and CF have also been detected along with TCE in the groundwater and subsurface of McClellan AFB. CF is known recalcitrant compound exhibiting slow transformation rates in the subsurface. 1,1,1 TCA can be abiotically converted to 1,1-DCE in the subsurface (Vogel and McCarty, 1987). CF and 1,1,1- TCA are of particular concern due to their toxicity and carcinogenicity. They are regulated by EPA to a maximum contaminant level of 0.1 mg CF/L and 0.2 mg 1,1,1-TCA/L (Cook, 1987; McCarty and Semprini, 1994).

The ability to stimulate microbes on methane, propane and butane was demonstrated in the microcosms constructed with McClellan subsurface solids and groundwater. Our study showed that McClellan subsurface appears to have a diverse microbial community, since we were successful in stimulating microbes on all the substrates tested. A lag period of about 20 days was observed before propane and butane uptake was observed. This was about twice the lag period of methane utilizers (10 days).

Indigenous microorganisms grown on propane were capable of transforming TCE, CF, and 1,1,1-TCA. Indigenous microorganisms grown on methane transformed TCE and CF, but did not transform 1,1,1-TCA. Butane-utilizers were not capable of cometabolizing any of the CAHs tested. Methane-utilizers exhibited highest transformation yields for TCE (0.07 mg TCE/mg methane), compared to about 0.05 mg TCE/mg propane). The propane-utilizers effectively transformed CF, TCE and 1,1,1-TCA. The initial results showed that propane appears to be more effective than methane in transforming CAHs mixtures.

C. INITIAL EVALUATION OF MICROBIAL ACTIVITIES AT EDWARD AFB

Methane, propane, and butane were also studied as potential cometabolic growth substrates with Edwards AFB subsurface solids and groundwater. Edwards AFB was included in our study since an in-situ field demonstration using toluene as a cometabolic substrate was being conducted at the site (McCarty et al., 1998). Thus in-situ delivery systems developed for the toluene demonstrations, might be used later used with methane, propane, or butane, as alternative substrates. Thus a field comparison to toluene as a cometabolic substrate could be performed if the microcosm results with the gaseous substrates were encouraging.

Methane-utilizers were stimulated after a lag time of about 20 days in the Edward microcosms. This lag time was about twice that observed in the McClellan microcosms. We had no success stimulating butane and propane utilizing microorganisms. The stimulated methane-utilizers were able to transform TCE. Their maximum transformation yield was about 0.01 mg TCE/mg methane. Thus, the transformation achieved was lower than achieved with the methane- utilizers that stimulated from the McClellan subsurface.

Jenal-Wanner and McCarty (1997) conducted Edward's microcosm studies using phenol and toluene and cometabolic substrates. Transformation yields calculated from their reported data were approximately 0.07 mg TCE/mg of toluene and 0.05 mg TCE/mg of phenol. Thus the toluene utilizers stimulated in the microcosms and later evaluated in the field were more effective towards TCE transformation than the methane-utlizers stimulated in our study.

D. INITIAL EVALUATION OF MICROBIAL ACTIVITIES AT MOFFETT AFB.

Moffett Federal Airfield (formerly the Moffett Naval Air Station), Mountain View, CA, was contaminated with various chlorinated aliphatic hydrocarbons (CAHs) including 1,1,1-TCA. Since 1985, there have been a number of studies at the site of aerobic cometabolism to transform CAHs. Methane phenol, toluene, ammonia and propane were tested for their abilities to cometabolize CAHs both in-situ and in microcosms (Hopkins et al., 1993; Semprini et al., 1993; Hopkins and McCarty, 1995).

In-situ field tests were performed using methane (Semprini et al., 1990; Semprini et al., 1991), phenol (Hopkins et al., 1993; Semprini et al., 1993; Hopkins and McCarty, 1995) and

toluene (Hopkins and McCarty, 1995) as cometabolic substrates. Microcosms studies were perform with phenol (Hopkins et al., 1993; Semprini et al., 1993), methane (Lazarone and McCarty, 1990; Semprini et al., 1993), propane (Lazarone and McCarty, 1990), toluene and ammonia (Hopkins et al., 1993). The studies verified that groundwater microorganisms can be stimulated by adding each primary substrate to groundwater in the presence of oxygen.

Table 2 presents the transformation of CAHs of interest by microbes grown on different substrates for the Moffett Subsurface. Phenol and toluene utilizers were more rapidly stimulated than methane and ammonia utilizers tested in Moffett Field microcosms and Field test. Phenol and toluene-fed microcosms degraded TCE and cis-DCE effectively. Methane-fed microcosms effectively degraded VC and trans-DCE and c-DCE and TCE to some extent. The ammonia fed microcosm was the least effective in degrading all CAHs. Propane-fed microcosms showed no propane utilization. No substrate was effective in transforming 1,1,1-TCA transformation.

Table 2. The transformation of chlorinated ethenes of interest by microbes grown on different substrates in Moffett Field microcosms and Field Tests

Substrate	**Type of study**	**CAHs transformation**				
		TCE	**cis-DCE**	**trans-DCE**	**VC**	**1,1-DCE**
Phenol	Field/Microcosm	✓✓✓	✓✓✓	✓✓	✓✓✓	✓
Toluene	Field/Microcosm	✓✓✓	✓✓✓	✓✓	✓✓✓	✓
Methane	Field/Microcosm	✓	✓✓	✓✓✓	✓✓✓	
Ammonia	Microcosm		✓	✓		
Propane	Microcosm [a]					

a No substrate utilization reported ✓ Some transformation was observed
✓✓✓ Very good transformation
✓✓ Good transformation

E. SUMMARY OF MICROBIAL ACTIVITIES AT THREE SITES

Table 3 summarizes our microcosm results for TCE, 1,1,1-TCA and CF transformation by microorganisms stimulated at the McClellan, Edward, and Moffett sites. The results of this screening demonstrated both the presence and absence (unable to stimulate) of microbes in the subsurface with abilities to grow on the cometabolic substrate of interest. The McClellan subsurface appears the most promising for stimulating microbes on the gaseous substrates tested. Methane and propane-utilizers stimulated from McClellan subsurface were capable of transforming both TCE and CF. The propane-utilizers could cometabolize 1,1,1-TCA and CF with high transformation yields. No transformation of CAHs was observed by butane-utilizers.

Table 3. Results from the microcosm screening studies of TCE, 1,1,1-TCA, and CF cometabolism by microbes grown on different gaseous cometabolic substrates using groundwater and aquifer solids from the McClellan, Edward, and Moffett sites

Air Force Base Site	Growth Substrate	Lag time before Substrate Utilization (days)	Maximum T_y for TCE	Maximum T_y for 1,1,1-TCA	Maximum T_y for CF
McClellan	Methane	10	0.069	0	0.024
	Propane	24	0.048	0.106	> 0.068
	Butane	20	0	0	0
Edwards	Methane	18	0.012	-	-
	Propane	N.A.	-	-	-
	Butane	N.A.	-	-	-
Moffett	Methane	-	-	-	-
	Propane	85	-	0.135	-
	Butane	80	-	0.044	-

T_y = transformation yields (g CAH/ g of Growth Substrate Used)
N.A : Complete substrate utilization was not achieved

The indigenous McClellan microorganisms were chosen for a detailed study, since they showed good transformation abilities for TCE, which a main contaminant of interest at the site. The McClellan site is a good candidate for treatment using gaseous cometabolic substrates using an gas sparging approach. The groundwater contamination is fairly deep at the site, and the vadose zone is fairly thick. Thus propane or methane might be injected along with air or oxygen into the groundwater to promote aerobic cometabolism in both the saturated zone, and the vadose zone. Since the site also contains mixtures of CAHs, the studies as concentrated on the transformation of CAH mixtures using single and mixed cometabolic substrates. Details of these studies are provided in Sections IV, V, and VI.

The Edward methane-utilizers showed some TCE transformation ability. The transformation of CF and 1,1,1-TCA were not tested, since these contaminants were not of interest at the Edwards site. The ability to stimulate propane and butane-utilizers was not observed in this study. Comparing the results of TCE cometabolism obtained with methane-utilizers obtained in this study, and those obtained with toluene-utilizers (Jenal-Wanner and McCarty, 1997), indicate that the toluene-utilizers were much more effective. Based on this result of our screening tests detailed studies of microbes stimulated from the Edwards site were not performed.

With Moffett subsurface solids and groundwater, we were able to stimulate propane and butane-utilizers with the significant lag time of about 80 days. This lag time was about four times as long as that observed in the McClellan microcosms. The long lag does raise concern about whether the microorganisms are actually indigenous, or could possible result from contaminant in the laboratory. Both propane and butane-utilizers that were stimulated were capable of cometabolize 1,1,1-TCA. Their abilities appear to be somewhat similar to those stimulated from the McClellan subsurface solids and groundwater.

Previous studies at the Moffett Field site have shown methane-utilizers and phenol-utilizers that were stimulated were not capable of cometabolically transforming 1,1,1-TCA. Thus a field demonstration at that site the cometabolism of 1,1,1-TCA would add to information for past field demonstrations. Since propane and butane-utilizers had long periods before effective biostimulation was achieved, the detailed work for the Moffett site focused on bioagumentation of butane and propane-utilizers and how this might be effective achieved under the prevailing nutrient conditions of the groundwater. The results of detailed bioagumentation studies are provided in Section III.

SECTION III

COMPARISON OF INDIGENOUS AND BIOAUGMENTED BUTANE AND PROPANE-UTILIZERS FOR TRANSFORMING 1,1,1-TRICHLOROETHANE IN MOFFETT FIELD MICROCOSMS.

A. INTRODUCTION

1,1,1-TCA is one of the volatile chlorinated compounds found most frequently in groundwater in the United States. It is a non-flammable solvent widely used in various industries and can be found in many household products. 1,1,1-TCA is considered relatively highly soluble, therefore it can spread quickly and easily in the subsurface. More than 200 sites on National Priorities lists were contaminated with 1,1,1-TCA (ATSDR 1990). Environmental Protection Agency (EPA) regulates 1,1,1-TCA level to be 0.2 ppm in drinking water and 18 ppm in rivers and streams. 1,1,1-TCA is relatively resistant to degradation in nature. It can be removed from groundwater by physical-chemical processes such as thermal regeneration and adsorption, but these processes only transfer 1,1,1-TCA from one medium to another without complete destruction. On the other hand, biological processes, which mineralize 1,1,1-TCA into harmless substances may be more practical.

1,1,1-TCA can be anaerobically transformed, but 1,1-DCA, its transformation product, is even more recalcitrant. It is also abiotically transformed to 1,1-DCE (which drinking water standard is 0.005 ppm) in water (Vogel and McCarty, 1987; Vogel and McCarty, 1987). Aerobic microorganisms can transform 1,1,1-TCA by cometabolism, yielding 2,2,2-trichloroethanol (Oldenhuis et al., 1989). This process involves oxygenase enzymes, which are produced by many aerobic microorganisms. Oxygenase enzymes are responsible for initiating the oxidation of primary substrates and transforming chlorinated compounds. Since both substrates have to compete for the same enzyme, the presence of a primary substrate can inhibit the transformation of a cometabolic substrate or vice versa. There are many types of oxygenase enzymes produced by various microorganisms. Each type of enzyme can have different abilities to degrade chlorinated compounds. Environmental conditions can also affect the type of enzymes stimulated. Methane monooxygenase enzymes, which are produced by methane-utilizers, can be categorized into 2 types, particulate (pMMO) and soluble (sMMO). sMMO can degrade a broader range of chlorinated compounds, but must be stimulated under copper limited conditions. pMMO does not need copper limited conditions, but can degrade fewer chlorinated compounds. Several substrates have shown an ability to stimulate microorganisms to cometabolize 1,1,1-TCA: methane (Broholm et al., 1990; Strand et al., 1990), butane (Kim et al., 1997; Kim et al., 1997) and propane (Keenan et al., 1993; Kim 1996).

The groundwater at Moffett Field Naval Air Station (Mountain View, CA) was contaminated with various chlorinated aliphatic hydrocarbons (CAHs), including 1,1,1-TCA (Roberts et al., 1990). Previous studies have demonstrated successful aerobic cometabolism of various CAHs both in-situ and in microcosm tests (Table 2). Phenol and toluene were good primary substrates for cometabolizing TCE and cis-DCE (Hopkins et al., 1993; Semprini et al., 1993; Hopkins and McCarty, 1995). Methane-utilizers were effective in degrading vinyl chloride (VC), trans-DCE and some TCE (Lazarone and McCarty 1990; Semprini et al., 1990; Semprini et al., 1991; Semprini et al., 1993). No cometabolic substrate tested, however, was effective toward 1,1,1-TCA cometabolism. Methane-utilizers at Moffett Field likely produced pMMO, which can not degrade 1,1,1-TCA, instead of sMMO (Semprini, 1997). Since the subsurface conditions at Moffett Field did not favor sMMO stimulation, the use of methane-utilizers for cometabolizing 1,1,1-TCA was diminished. Therefore, butane and propane were selected in this study as potential primary substrates for cometabolizing 1,1,1-TCA under Moffett Field conditions.

The information on butane-utilizers is limited. Soil microcosm studies showed that 1,1,1-TCA concentrations as high as 2400μg/L in aqueous solution can be transformed completely (Kim et al., 1997; Kim et al., 1997b). The oxygenase enzyme systems of butane-utilizers have not been studied extensively. It is possible that butane-utilizers can express more than one type of oxygenase enzyme, some of which may not be able to transform 1,1,1-TCA. A soil microcosm study of butane-utilizers stimulated from the McClellan subsurface was successful in degrading butane, but failed to transform 1,1,1-TCA (Section IV).

Propane-utilizers have also demonstrated ability to transform 1,1,1-TCA (Keenan et al., 1993; Kim, 1996). Propane-utilizers express propane monooxygenase enzyme (PMO), which has a broad substrate range (Wackett et al., 1989). PMO is likely responsible for propane oxidation as well as 1,1,1-TCA transformation. As a result, competitive inhibition was observed. Propane strongly inhibits 1,1,1-TCA transformation (Keenan et al., 1993) and high concentrations of 1,1,1-TCA could slow down propane degradation rate (Section IV)

Occasionally, the indigenous microorganisms cannot degrade the target contaminant due to a lack of appropriate enzymes, the absence of appropriate populations or low number of populations (Vogel, 1996). Microorganisms enriched in the laboratory, which have an ability to cometabolize the target contaminant, can potentially be inoculated into the specific site. This process is known as bioaugmentation. Various studies have shown that bioaugmentation is effective in transforming the target contaminant, improving transformation efficiency and reducing long lag times (Frosyth et al., 1995; Hinchee et al., 1995; Mayotte et al., 1996; Munakata-Marr et al., 1996; Vogel 1996; Dybas et al., 1997; Munakata-Marr et al., 1997; Munakata-Marr et al., 1997). One of the major concerns in bioaugmentation is creating and maintaining the appropriate environment for the inoculated strain to grow and survive.

A batch-fed microcosm method was selected to evaluate the potential of using butane and propane as primary substrates to drive the cometabolism of 1,1,1-TCA. Indigenous butane and propane-utilizers from the Moffett Field subsurface were stimulated and their performances were compared with bioaugmented strains. Since inoculated strains were grown in mineral salt nutrient in the laboratory, their adjustment to less nutrient abundant environments was evaluated. This research included a study on the necessity and effect of nutrient addition along with bioaugmentation. A PCR method followed by DNA separation by electrophoresis was used to evaluate the microbial population stimulated in various conditions to support the microcosm observations.

B. MATERIALS AND METHODS

1. Chemicals

Propane (10% in Nitrogen) and butane (10.2% in Nitrogen) were purchased from Aldrich Chemical Co. (Milwaukee, WI). 1,1,1-TCA (99.9% {GC} grade) was also purchased from Aldrich Chemical Co. GeneAmp® PCR core reagents (Ampli Taq DNA Polymerase, dNTPs, 10X PCR buffer II and $MgCl_2$) were purchased from Perkin Elmer (Foster City, CA).

2. Microcosm Preparation and Operation

Batch-fed microcosms were constructed using 125-ml amber serum bottles (Wheaton Glass co., Millville, NJ.). The bottles were filled with 15 ml of aquifer material and 50 ml of groundwater from the Stanford Test Facility at Moffett Field, leaving 60ml of air headspace in the bottles. The bottles were crimped sealed with a Teflon™/butyl rubber septum to accommodate frequent sampling. Butane or propane was added to each microcosm by gaseous addition to achieve a total mass of 4.5 mg. A stock solution of saturated 1,1,1-TCA in water was added to each microcosm. The microcosms were shaken for 30 min to equilibrate the concentrations in aqueous and gas phase, then sampled for the initial concentrations. Microcosms were incubated at room temperature on a shaker table at 100 rpm. Substrate and 1,1,1-TCA concentrations were monitored daily. Pressure was re-equilibrated daily to replace the oxygen in the headspace.

Propane and butane enrichment growth reactors were set up in 275-ml bottles. The bottles were filled with 175 ml of mineral salt growth media. 5 ml of suspended liquid taken from soil microcosms was added to inoculate each growth reactor. Butane or propane was added by gaseous addition to each bottle to yield 20% v/v in headspace. The growth reactors were placed on a shaker table to 100 rpm in 30°C constant temperature room. Pressures were equilibrated daily with pure oxygen and air. After about 70% of butane or propane were consumed, the cells were harvested for the bioaugmentation studies.

3. Bioaugmentation

Butane and propane-utilizers for bioaugmentation were acquired from enrichments obtained from the Hanford DOE site (Richland, Washington) (Kim 1996) grown on mineral salts medium at an optical density (OD_{600}) of 1.3 to 1.9. The cells were washed with groundwater to rinse away growth media before adding them to the microcosms.

Bioaugmentation Study: A set of microcosms was prepared as described above by adding 15 ml of aquifer material and 50 ml of groundwater (Table 4). Two bottles were fed with butane and 1,1,1-TCA (B1 and B2) and the other two were fed propane and 1,1,1-TCA (P1 and P2). The total mass to 1,1,1-TCA added initially was approximately 30 μg. One bottle with only 1,1,1-TCA and groundwater served as a control. After 57 days (with no substrate consumption observed), microcosms B2 and P2 were bioaugmented with butane and propane-utilizers, respectively. The microcosms were sampled daily until substrate utilization and 1,1,1-TCA transformation stopped. Then, 60% of groundwater was exchanged with fresh groundwater to supply nutrients and prevent transformation by-products from building up. 1,1,1-TCA mass was gradually increased in each batch until the maximum transformation yield was reached.

Table 4 Treatment variations in microcosms B1, B2, P1 and P2

Microcosm #	Primary Substrate	1,1,1-TCA	Bioaugmentation
B1	Butane	✓	—
B2	Butane	✓	✓ with butane-utilizers
P1	Propane	✓	—
P2	Propane	✓	✓ with propane-utilizers

After 440 days of repeated stimulation, 5-ml liquid of each microcosms was inoculated into growth reactors as described earlier. The enrichments were grown on mineral salt medium for 30 days before inoculated into new soil microcosms. The new microcosms were fed with butane or propane and 1,1,1-TCA. Substrates and 1,1,1-TCA consumptions were monitored to see if the enrichments have become nutrient dependent.

4. Nutrient Effect Study

To assess the effects of nutrient addition, three sets of augmented microcosms were set up with varying ratios of groundwater and mineral salt media (Table 5). Each set contained 2 microcosms. The first set of microcosms was set up with 5 ml aquifer material and 50 ml groundwater and bioaugmented with 5 mg cells. Second and third sets were set up similarly except 5% and 50% mineral salt media (media composition is given in Appendix A) in groundwater were added to second and third set subsequently. The same study was done in parallel for both butane and propane-utilizers.

Table 5 Treatment variations in microcosms B3-B5, BT3-BT5, P3-P5 and PT3-PT5

Microcosm #	Primary Substrate	Growth Media	1,1,1-TCA
B3	Butane	—	—
B4	Butane	5%	—
B5	Butane	50%	—
BT3	Butane	—	✓
BT4	Butane	5%	✓
BT5	Butane	50%	✓
P3	Propane	—	—
P4	Propane	5%	—
P5	Propane	50%	—
PT3	Propane	—	✓
PT4	Propane	5%	✓
PT5	Propane	50%	✓

- 100% Groundwater

Butane, propane and 1,1,1-TCA were added in the same fashion as in the bioaugmentation study. After several groundwater exchanges, the microbial population differences among each microcosm were determined by polymerase chain reaction (PCR) followed by DNA separation by electrophoresis.

After several groundwater exchanges, 5-ml liquid from B3, B4, B5, BT3, BT4 and BT5 were inoculated in 100% media microcosms without aquifer solids. These microcosms were fed with butane without 1,1,1-TCA addition. After 1 month, 1,1,1-TCA was added to these butane-utilizers to test their ability to transform 1,1,1-TCA.

5. Analytical Procedures

1,1,1-TCA was measured by injecting a 100-µl headspace sample from headspace and injecting into a Hewlett Packard 5890 gas chromatography connected to an electron capture detector (ECD). Butane and propane sampling was similar, except a flame ionization detector (FID) was used. The headspace oxygen concentration was measured on a Fisher Model 25Vgas partitioner equipped with a thermoconductivity detector, with helium as a carrier gas. 100-µl sample from headspace was injected into the gas partitioner. Nitrate analysis was performed with a Dionex series 4000I Ion Chromatography coupled to an ion exchange column (Dionex IonPac AS4A). 1,1,1-TCA, butane, propane, oxygen and nitrate concentrations were determined by comparing peak areas of samples with those of external standards curves.

6. Polymerase Chain Reaction

6.1 DNA purification: Samples (500 μL) were obtained from well shaken microcosms for PCR analysis. The microorganisms cells were ruptured and DNA was released from the cells by a direct lysis method (Current Protocols, 1994). DNA was extracted from cell material and sediments by phenol-chloroform and then precipitated in ethanol. The DNA was then dissolved in water and purified by electrophoresis in 0.75% agrose gel. The gels that contain purified DNA were cut into a plug and immersed in water to diffuse DNA into water.

6.2 PCR (Polymerase Chain Reaction): The PCR procedure was modified from Current Protocols in Molecular Biology, 1994. The purified DNA was combined with PCR reaction mixture, which contains: *Taq* DNA polymerase, oligomer primers, deoxynucleotides (dNTPs), reaction buffer and magnesium ions. The mixtures were then place into thermocycler (Ericomp Power Block™ Easy Cycler™ series). The samples were heated to 95°C for 45 sec, cooled down to 45°C for 45 sec and heated to 72°C for 60 sec. The cycle was repeated 10 times. Then another cycle which consisted of heating to 92° C for 45 sec, cooling to 45°C for 45 sec and heating to 72°C for 90 sec was repeated 20 times. After this process DNA fragments were separated by electrophoresis at 85 volts in 1.2 % agrose.

C. RESULT AND DISCUSSION

1. Indigenous versus augmented butane-utilizers

Figure 7 presents butane utilization and 1,1,1-TCA transformation in microcosms B1 and B2 for the first 94 days of stimulation. Microcosms B1 and B2 were fed with butane to stimulate indigenous butane-utilizers and challenged with 1,1,1-TCA. After 57 days, no substrate utilization was observed, indicating that butane-utilizers were not stimulated in either microcosm. Microcosm B2 was then inoculated with butane-utilizing enrichment described earlier. On day 60, the butane mass in inoculated microcosm B2 started to rapidly decrease, indicating butane utilization. As the butane was being utilized, 1,1,1-TCA was also being transformed. 1,1,1-TCA transformation rate was slow in the beginning, but increased dramatically after a significant amount of butane was consumed.

Microcosm B1 started to degrade butane after 80 days, indicating that indigenous butane-utilizers were present. Butane was completely utilized, but no 1,1,1-TCA was transformed. The results suggest that butane-utilizers stimulated in B1 initially lacked the ability to transform 1,1,1-TCA. The augmented microcosm (B2) however, effectively transformed 1,1,1-TCA. The lag time observed in microcosm B1 was very long, therefore, the possibility of microbial contamination of the microcosm could not be

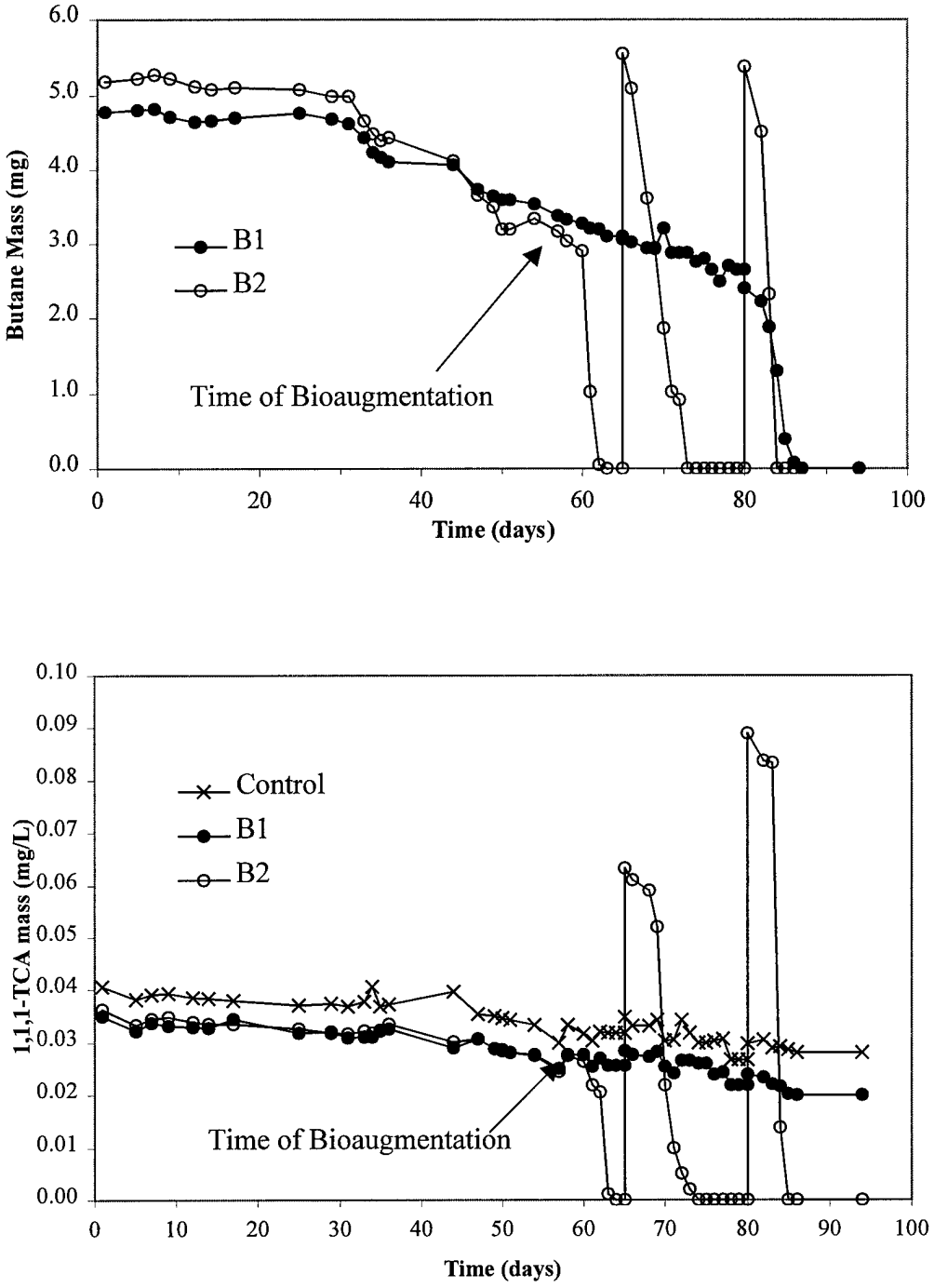


Figure 7 Butane utilization and 1,1,1-TCA transformation of microcosms B1 and B2 after 94 days of stimulation.

entirely ruled out. However, the differences in ability to degrade 1,1,1-TCA of microcosms B1 and B2 seem to indicate that indigenous microbes with limited 1,1,1-TCA transformation were stimulated. Therefore, the "indigenous" microcosm in this study refers to microcosm that was stimulated without bioaugmentation, although we were not certain that they were truly indigenous.

1,1,1-TCA mass added to microcosm B2 was gradually increased with successive additions of butane stimulation. Microcosm B2 continued to effectively transform 1,1,1-TCA. The transformation was slow when the butane mass was high. After a considerable amount of butane was consumed, 1,1,1-TCA transformation accelerated, indicating that 1,1,1-TCA transformation was inhibited by butane.

Figures 8 and 9 shows the responses of microcosm B1 and B2 after 100 days of stimulation. Microcosm B1 started to show some 1,1,1-TCA transformation around day 130. 1,1,1-TCA transformation was limited initially. After 190 days of stimulation, the 1,1,1-TCA transformation improved to over 90%.

Table 6 presents transformation yield of indigenous and bioaugmented butane-utilizers at three time periods. Transformation yields of microcosm B1 slowly improved. Microcosm B2 transformation yields decreased after repeated stimulations. After 340 days, the transformation yields in B1 and B2 were almost the same.

Table 6 Transformation yields of microcosms B1 and B2 at different time periods

Microcosm	Transformation Yield (mg 1,1,1-TCA/ mg butane)								
	Day 0 to190			Day 190 to 340			Day 340 to 440		
	Min	Max	Aver.	Min	Max	Aver.	Min	Max	Aver.
B1	0.0026	0.0048	0.0035	0.002	0.05	0.03	0.04	0.044	0.042
B2 (inoculated)	0.006	0.16	0.07	0.003	0.13	0.077	0.033	0.041	0.041

Microcosm B2 continued to transform 1,1,1-TCA completely throughout the first 180 days of stimulation (Table 6). The highest 1,1,1-TCA mass, which was transformed completely, was 0.71 mg (or 8.31 mg/L aqueous concentration). The maximum transformation yield up until day 190 was 0.16 mg 1,1,1 TCA/mg butane for microcosm B2.

After 220 days, 1,1,1-TCA mass added to microcosm B2 was raised to 1 mg (11.7 mg/L) (Figure 9). Only 20% of 1,1,1-TCA were transformed. From day 190 to day 340 1,1,1-TCA mass fed to the microcosm B2 ranged from 0.72 to 1.037 mg. 1,1,1-TCA was partially transformed during this period, (average 53%) (Table 7). The average transformation yield was 0.13 mg 1,1,1-TCA/mg butane (Table 6). Butane was completely degraded but the ability to transform 1,1,1-TCA decreased after exposure to

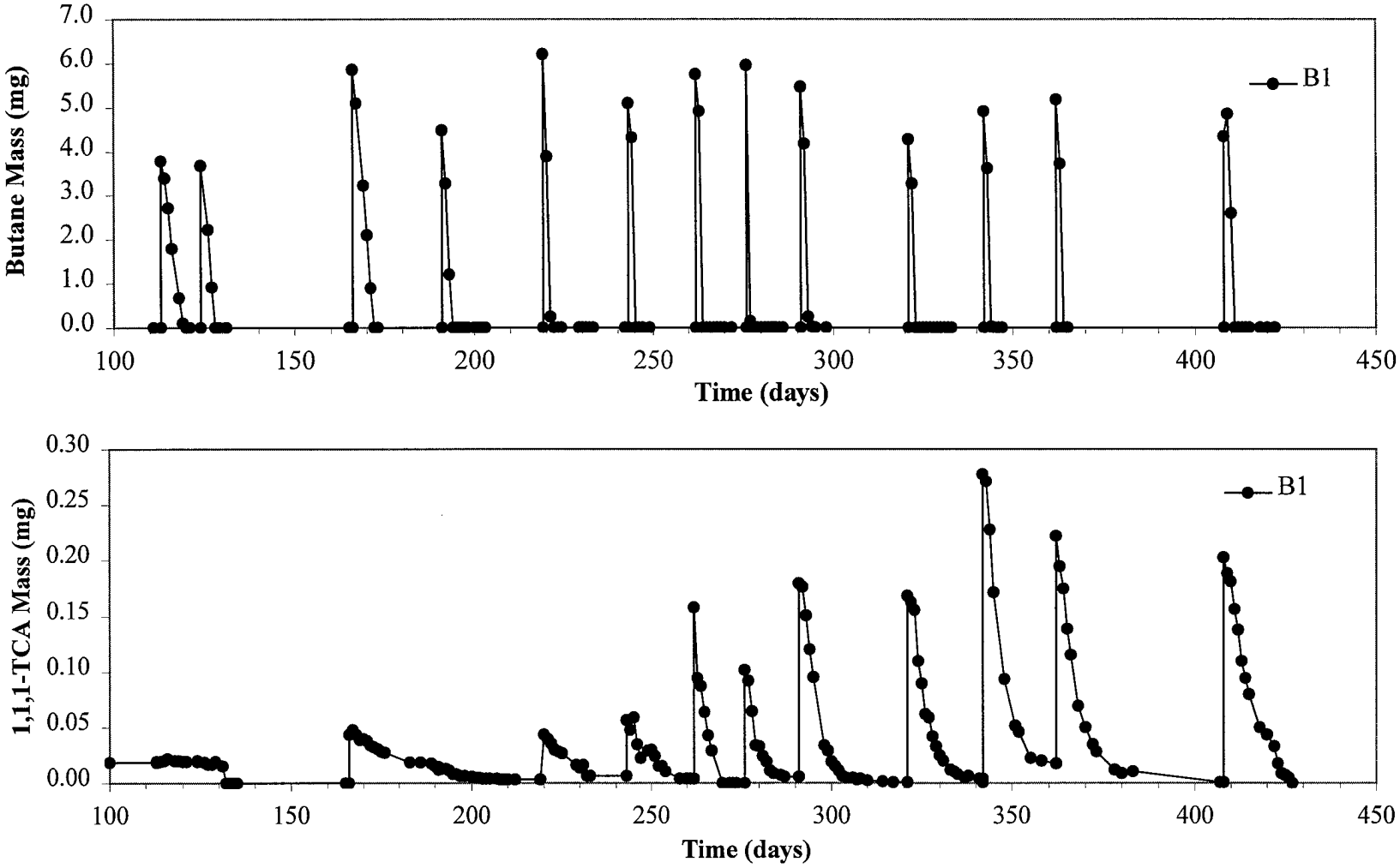


Figure 8 Butane utilization and 1,1,1-TCA transformation of microcosm B1 from 100 to 420 days

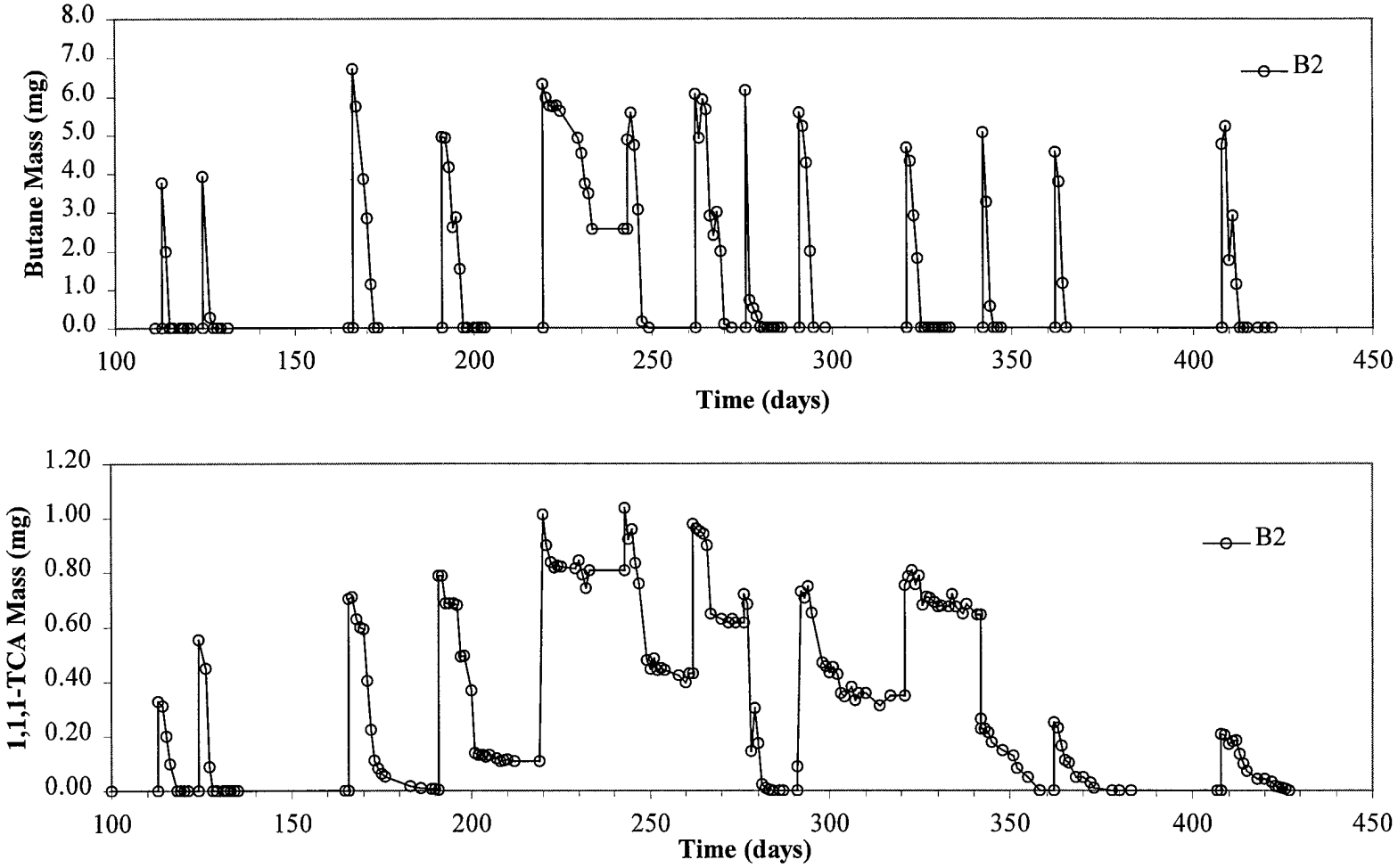


Figure 9 Butane utilization and 1,1,1-TCA transformation of microcosm B2 from 100 to 430 day

high 1,1,1-TCA concentrations. After 340 days, the 1,1,1-TCA mass was reduced to 0.27 mg (3.16 mg/L), which resulted in an increase of 1,1,1-TCA transformation efficiency to 99%.

Table 7 summarizes 1,1,1-TCA transformation efficiency at different time periods, while Table 8 summarizes the average time required for the substrate utilization and 1,1,1-TCA transformation. Microcosm B1 improved transformation efficiency and transformation rate after repeated stimulations. Butane degradation in microcosm B2 was fast and the efficiency observed was highest during the first 190 days. When the 1,1,1-TCA mass was raised to 1 mg/L, the butane degradation slowed down and the transformation efficiency decreased to 53%. After 1,1,1-TCA mass was decreased, transformation efficiency and butane degradation rate improved.

Table 7 Mass of 1,1,1-TCA added and percentage of 1,1,1-TCA transformed in microcosms B1 and B2 over different time periods.

Microcosm	1,1,1-TCA mass added (mg)			1,1,1-TCA transformed (%)		
	Day 0 - 190	Day 190-340	Day 340-440	Day 0 to 190	Day 190-340	Day 340-440
B1	0.019-0.047	0.018-0.179	0.202-0.277	40% (0-100%)	90.4% (82-99%)	96% (93-100%)
B2 inoculated	0.027-0.553	0.720-1.037	0.203-0.248	100%	53% (20-100%)	99% (98-100%)

Table 8 Average time required for the substrate utilization and 1,1,1-TCA transformation of microcosms B1 and B2 over different time periods

Microcosm	Butane-utilization (days)			1,1,1-TCA transformation (days)		
	Day 0 - 190	Day 190-340	Day 340-440	Day 0 - 190	Day 190-340	Day 340-440
B1	6.75	2.71	3.7	15.5	14.5	20
B2	5.83	9	4.7	7.5	10.57	16

1,1,1-TCA transformation continued after butane-utilization ceased, indicating that the enzymes were present and active after substrate utilization had stopped (Figure 10). Figure 10 compares 1,1,1-TCA transformation rates with butane utilization rates. At similar concentrations, both indigenous and bioaugmented butane-utilizers had slower 1,1,1-TCA transformations rates when the butane concentration were high and increased as a butane was consumed. The results indicated that butane inhibited 1,1,1-TCA transformation.

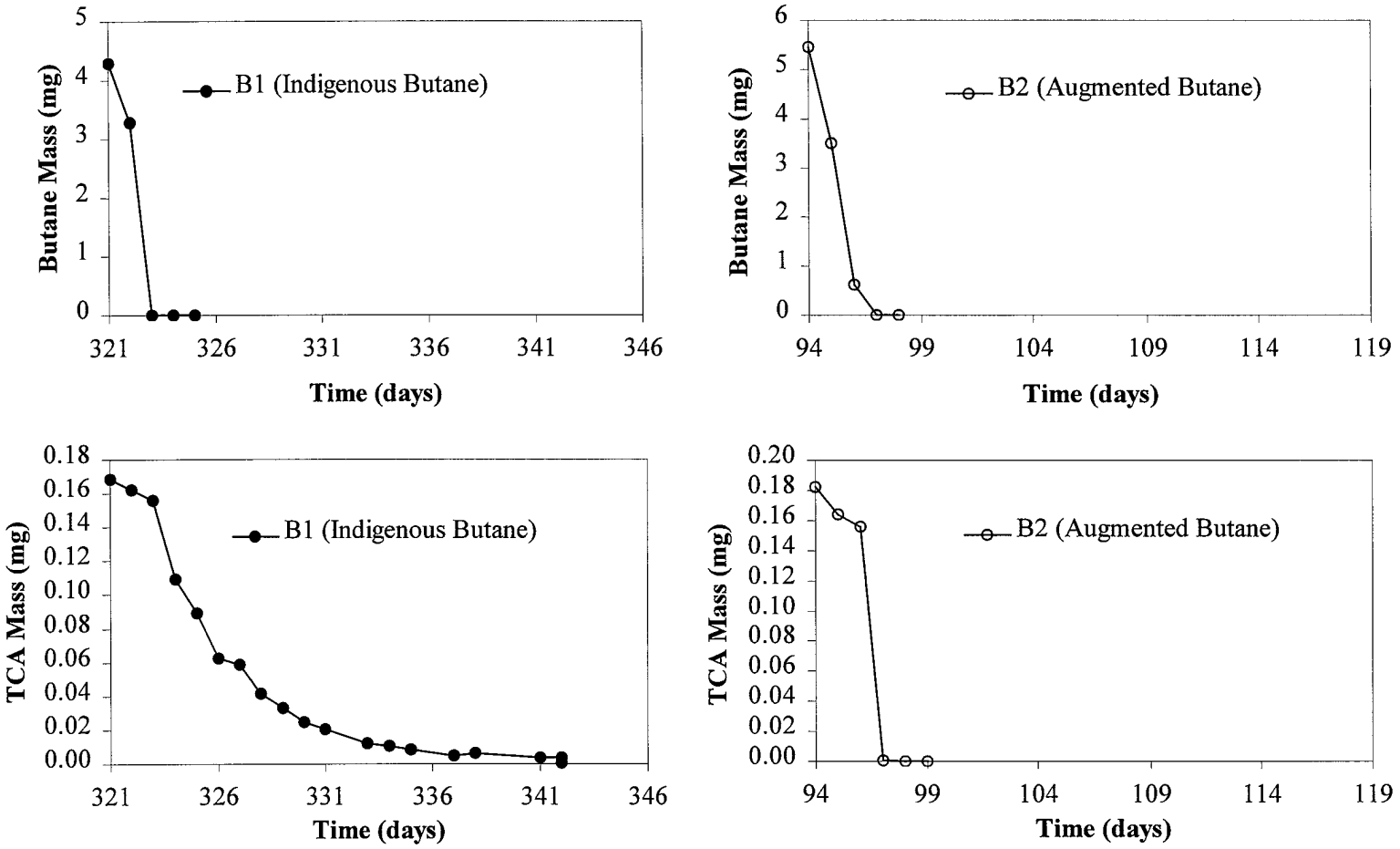


Figure 10 Butane utilization rate and 1,1,1-TCA transformation in microcosms B1 and B2 at similar 1,1,1-TCA concentrations

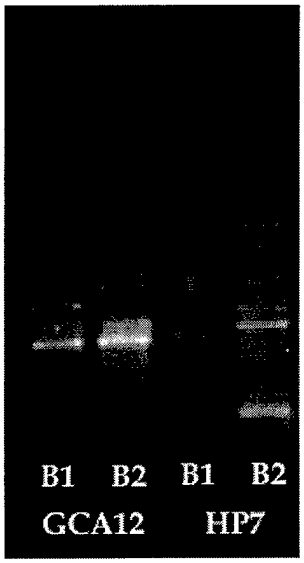


Figure 11 DNA fingerprints of butane-utilizers obtained from microcosms B1 and B2 on day 440.

Figure 11 shows the DNA fingerprints from butane-fed microcosm B1 and B2 after 440 days. The PCR was performed with two different primers, GCA12 and HP7. The DNA fragments in the gel showed similar bands sizes in both samples, suggesting that the populations in both microcosms were similar. It was possible that the indigenous population was predominant in both microcosms at the time tested. Bioaugmented microcosms showed higher activity than indigenous microcosm initially, but at 440 days, both indigenous and bioaugmented microcosms showed similar 1,1,1-TCA transformation activities and DNA fingerprints. This may indicate that the indigenous population had outcompeted the bioaugmented populations. Unfortunately, microbial samples for PCR analysis were not available from the early stages of this study to show if different mixed populations existed in microcosms B1 and B2.

2. Indigenous vs. augmented propane-utilizers

Figure 12 shows propane degradation and 1,1,1-TCA transformation data for microcosms P1 and P2 for the first 140 days of stimulation. Microcosm P2 was inoculated with propane-utilizing enrichments from Hanford DOE site. The inoculated microcosms, however, did not show propane consumption until day 80, 23 days after bioaugmentation. Upon stimulation, effective propane uptake was achieved, but no significant 1,1,1-TCA transformation was not observed. On day 94 propane was fed to microcosm P2 for a second time and it was effectively utilized. Some 1,1,1-TCA transformation was observed (12 μg). The indigenous strains in microcosm P1 started to utilize propane on day 85. Effective propane uptake was achieved and approximately 12 μg of 1,1,1-TCA was transformed.

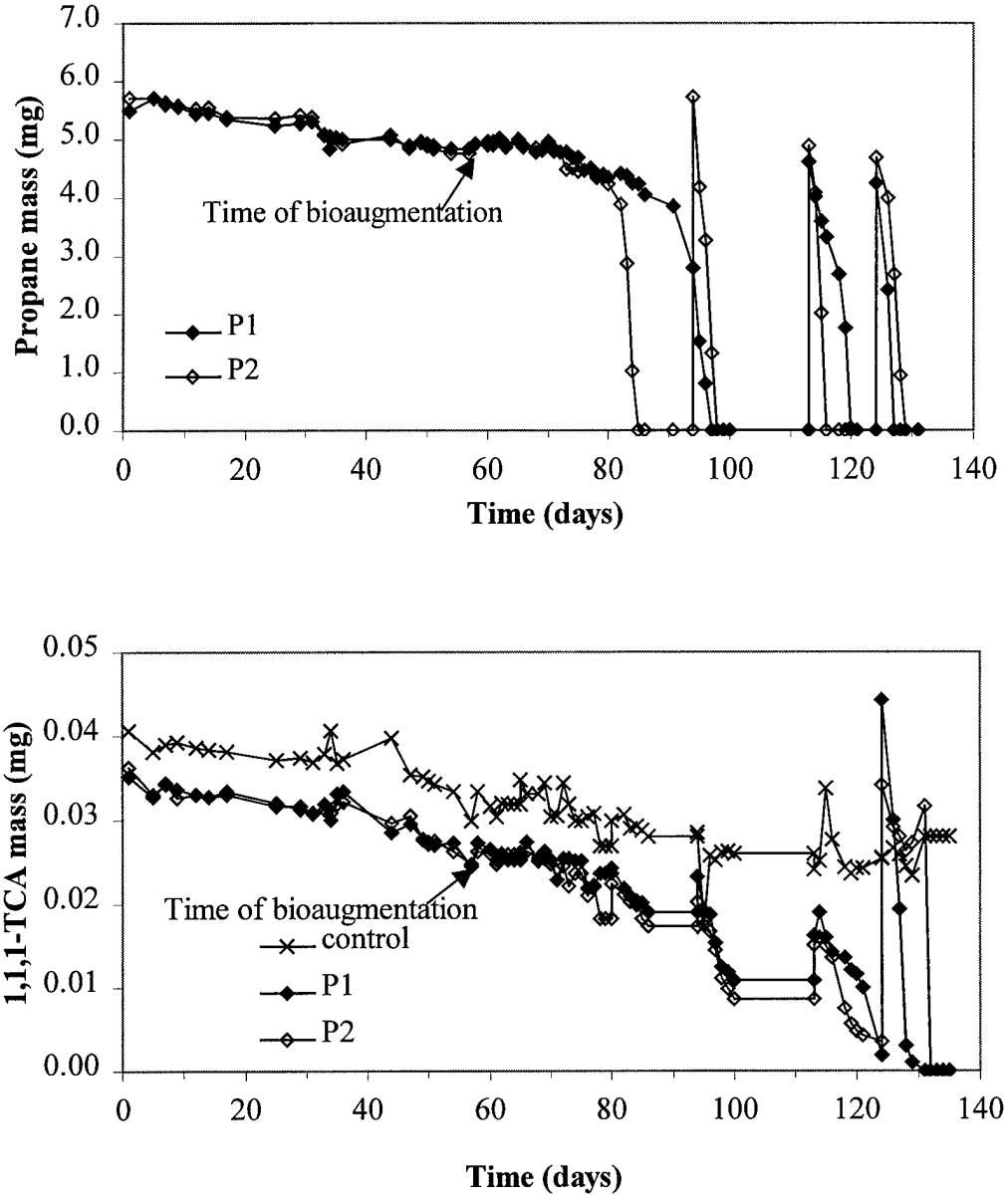


Figure 12 Propane utilization and 1,1,1-TCA transformation in microcosms P1 and P2.

Figure 13 shows P1 and P2 data from 160 to 430 days as 1,1,1-TCA mass was gradually increased with each of propane respike. Complete transformation of 1,1,1-TCA was observed up to day 323 upon raising the 1,1,1-TCA mass in the P2 microcosm to 0.78 mg (9.1 mg/L aqueous concentration) and only 70% of 1,1,1-TCA was transformed (Table 9). Both propane-utilizing microcosms remained active for more than 430 days of stimulation.

Table 9 1,1,1-TCA transformation efficiency of microcosms P1 and P2

Microcosms	1,1,1-TCA mass added (mg)	Transformation efficiency (%)
P1	0.012-0.54	100%
	0.401-0.737	86-97% (average 94%)
P2	0.012-0.76	100%
	0.806	70%

The highest 1,1,1-TCA concentration reduced the transformation efficiencies of both propane-utilizing microcosms. The similar results were obtained with the butane-utilizers. Both of the microcosms, P1 and P2, showed good long-term efficiencies, remaining active and expressing effective transformation yields for more than 340 days after stimulation. Microcosms P1 and P2 had similar lag times and long-term responses, e.g. transformation yields, in every time period (Table 10 and Figure 12-13). Figure 14 shows DNA fingerprints from microcosms P1 and P2 after 440 days using two different primers. DNA fingerprints showed the similar band sizes, which indicated that mixed populations of both microcosms were similar at the time tested.

It is possible that the indigenous propane-utilizers were stimulated in both microcosms. The augmented strains may have been active initially but indigenous propane-utilizers may have eventually dominated the microcosms. Unfortunately, microbial samples from the earlier stages were not available for PCR analysis to determine if changes occurred during the course of the tests.

Table 10 Transformation yields of microcosms P1 and P2

Microcosms	Lag Time (Days)	Transformation Yields (mg 1,1,1-TCA/mg propane)		
		Minimum	Maximum	Average
P1	85	0.0096	0.135	0.069
P2	80*	0.0075	0.14	0.066

*23 days lag time after bioaugmentation.

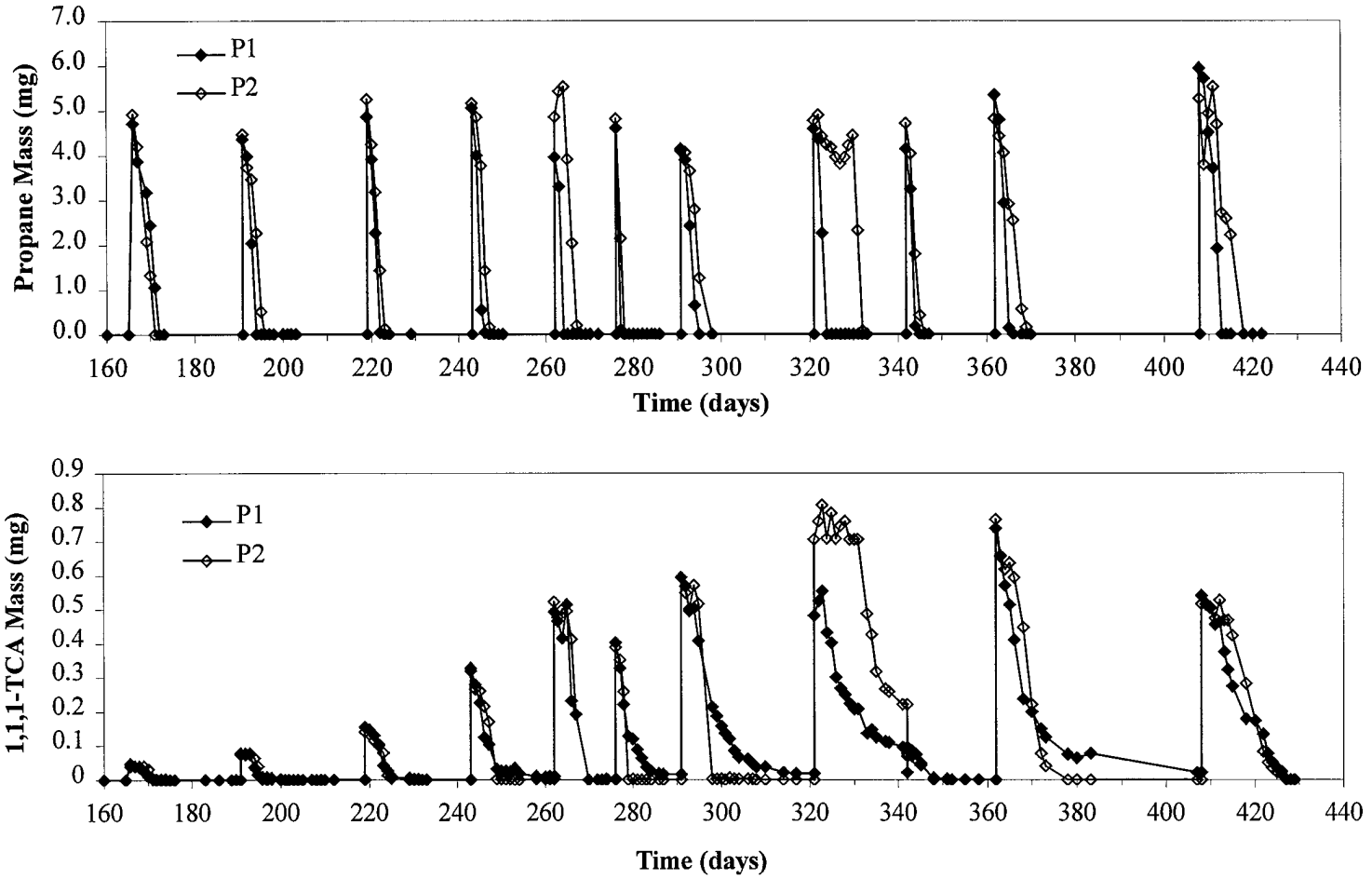


Figure 13 Propane utilization and 1,1,1-TCA transformation in microcosms P1 and P2 from 160 to 430 day.

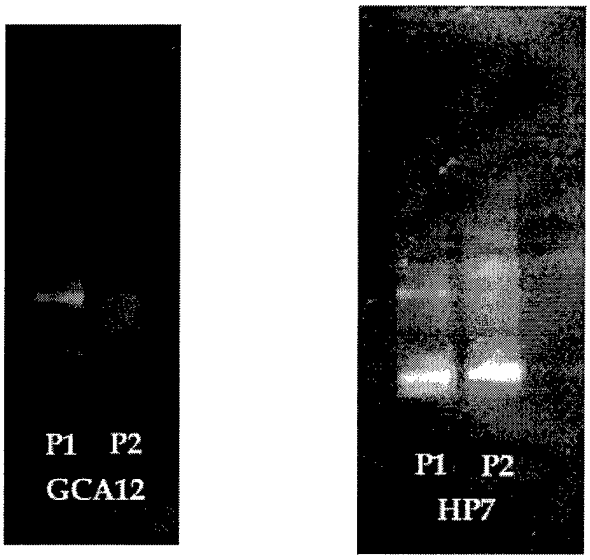


Figure 14 DNA fingerprints of propane-utilizers obtained from microcosms P1 and P2 on day 440

Figure 15 compares the 1,1,1-TCA transformation rates with propane utilization rates of indigenous and bioaugmented propane-utilizers at similar concentration conditions. The 1,1,1-TCA transformation rate was slow when the propane mass was high and as propane was depleted, 1,1,1-TCA transformation rate increased. 1,1,1-TCA transformation, therefore, appeared to be inhibited by the presence of propane in both indigenous and bioaugmented microcosms. Propane transformation may have also been inhibited by high concentrations of 1,1,1-TCA.

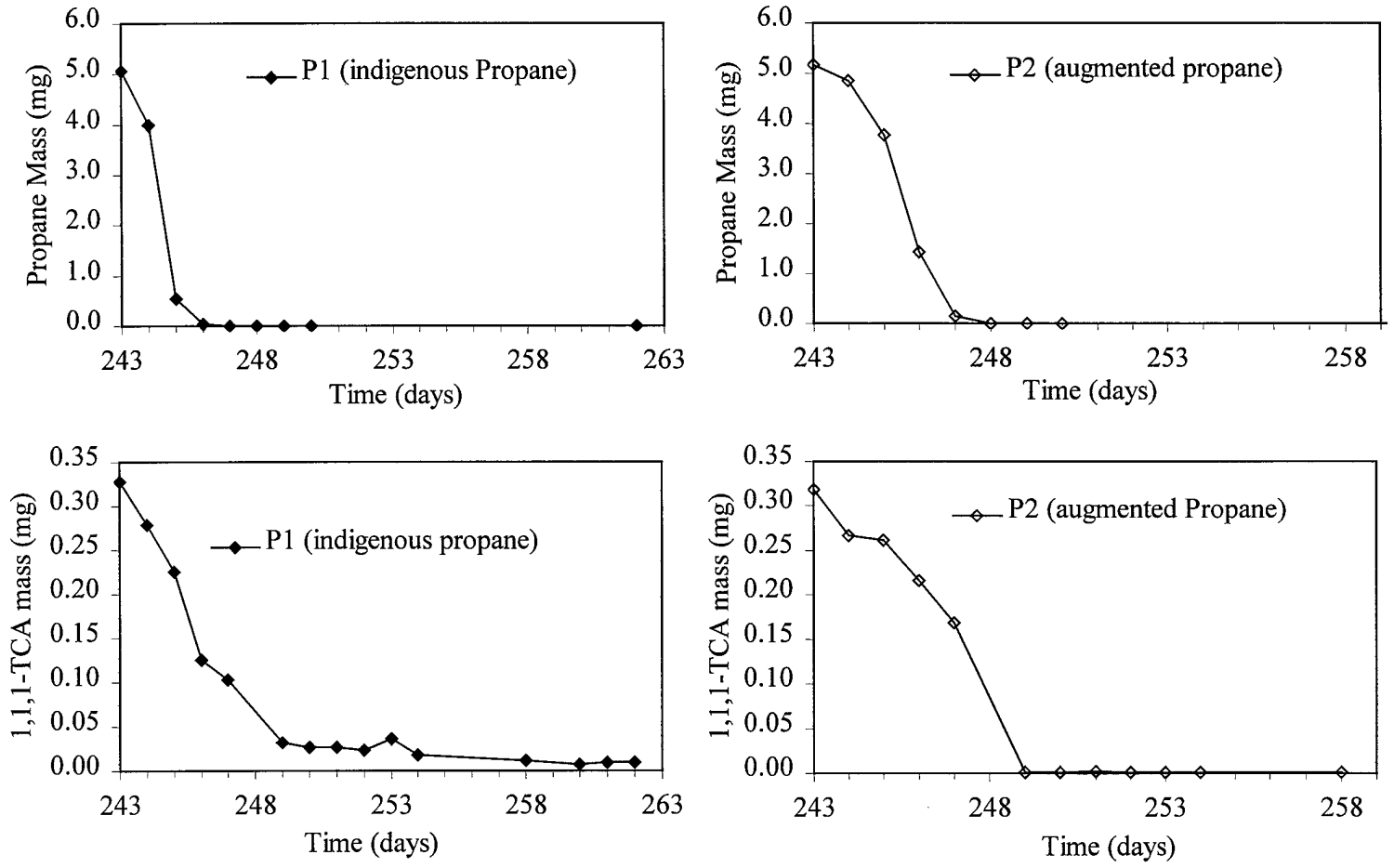


Figure 15 Propane utilization and 1,1,1-TCA transformation in microcosms P1 and P2 at similar 1,1,1-TCA concentrations.

3. Comparison of Butane and Propane-utilizers

Figure 16 shows transformation yields of butane and propane-utilizers at different stimulation periods. Microcosm B2, the bioaugmented butane-utilizers, had high transformation yields in the beginning (day 1 to 340), but the yields eventually decreased. Microcosms B1, P1 and P2 had low transformation yields in the beginning but increased with prolonged treatments. The propane-utilizers displayed more consistency 1,1,1-TCA transformation ability than the butane-utilizers. Propane-utilizers, therefore, appear to be better candidates for 1,1,1-TCA treatment under Moffett Field subsurface conditions.

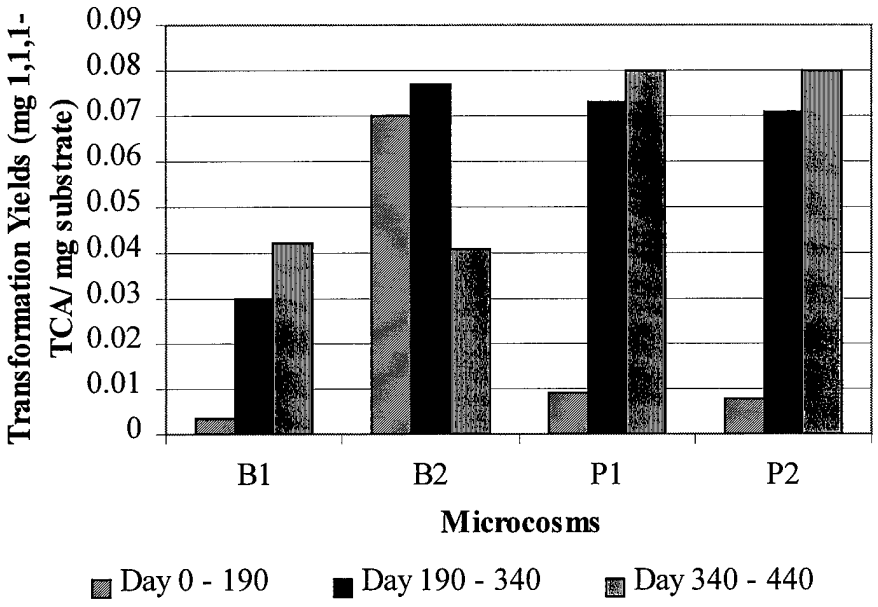


Figure 16 Comparison of transformation yields of microcosms B1, B2, P1 an dP2 at different time periods.

Attempts were made to repeat the bioaugmentation of new Moffett Field microcosms with butane and propane-utilizers. The bioaugmented microcosms did not show butane or propane degradation (data not shown). The bioaugmented cultures were taken from the enrichment growth reactors 150 days after the first inoculation. A shift in the microbial population of the growth reactors was suspected, since the enrichments were grown in mineral salt medium for a long time. Strains that grew well on the mineral media may become likely predominated in the enrichment, and these strains now failed in the nutrient limited groundwater microcosms. A study, therefore, designed to determine whether media addition was required to achieve successful bioaugmentation.

4. Nutrient Studies with Butane-Utilizers

The design of the nutrient addition experiments for the bioaugmentation tests are presented in Table 5. Figure 17 and 18 showed butane consumption and 1,1,1-TCA transformation of augmented butane-utilizers with different percentages of mineral media addition. Microcosms B3 and BT3, which did not contain media, showed the slowest rate of butane utilization. Microcosms B5 and BT5, with the highest percentages of media (50%), had the fastest rate of butane utilization. BT5 had the fastest 1,1,1-TCA transformation rate. 1,1,1-TCA transformation rates were positively correlated with butane consumption rates. 1,1,1-TCA transformation appeared to be slightly inhibited by butane. Each pair of microcosms; B3 and BT3; B4 and BT4; B5 and BT5 had similar butane utilization rates, showing that, at 1,1,1-TCA mass up to 0.3 mg (aqueous concentration 3.51 mg/L), the presence of 1,1,1-TCA did not affect the microbes' abilities to degrade butane.

With repeated additions of butane (Figure 18) with increasing 1,1,1-TCA concentrations, the butane utilization rates in microcosms B3 and BT3 decreased. 1,1,1-TCA transformation in microcosm BT3 also decreased, and after 60 days, and no 1,1,1-TCA transformation was observed. Microcosms B4 and BT4 with 5% media showed the similar responses, but with faster butane utilization rates than B3 and BT3. The 1,1,1-TCA transformation rate in microcosm BT4 also decreased, and on day 105, less than 10% of 1,1,1-TCA was transformed. The results indicated that bioaugmented enrichments needed mineral media supplements to maintain 1,1,1-TCA transformation efficiencies. 5% mineral salt was inadequate for prolonged treatments. Microcosms B5 and BT5 displayed high activities throughout the study, indicating that 50% mineral salt supplement helped maintain the transformation efficiencies.

The nutrient study was conducted 210 days after initial bioaugmentation study (Figure 7 and 8). Butane-utilizers were obtained from the same growth reactors. The butane-utilizers in the first bioaugmentation study (Figure 7 and 8) effectively transformed 1,1,1-TCA without mineral media addition. Bioaugmentation at the later time required nutrient addition. One possible is that the enrichments grown in mineral media selected for populations that performed well in mineral media. When these enrichments were used in the bioaugmentation study, only the microcosms with high amendments of media performed well.

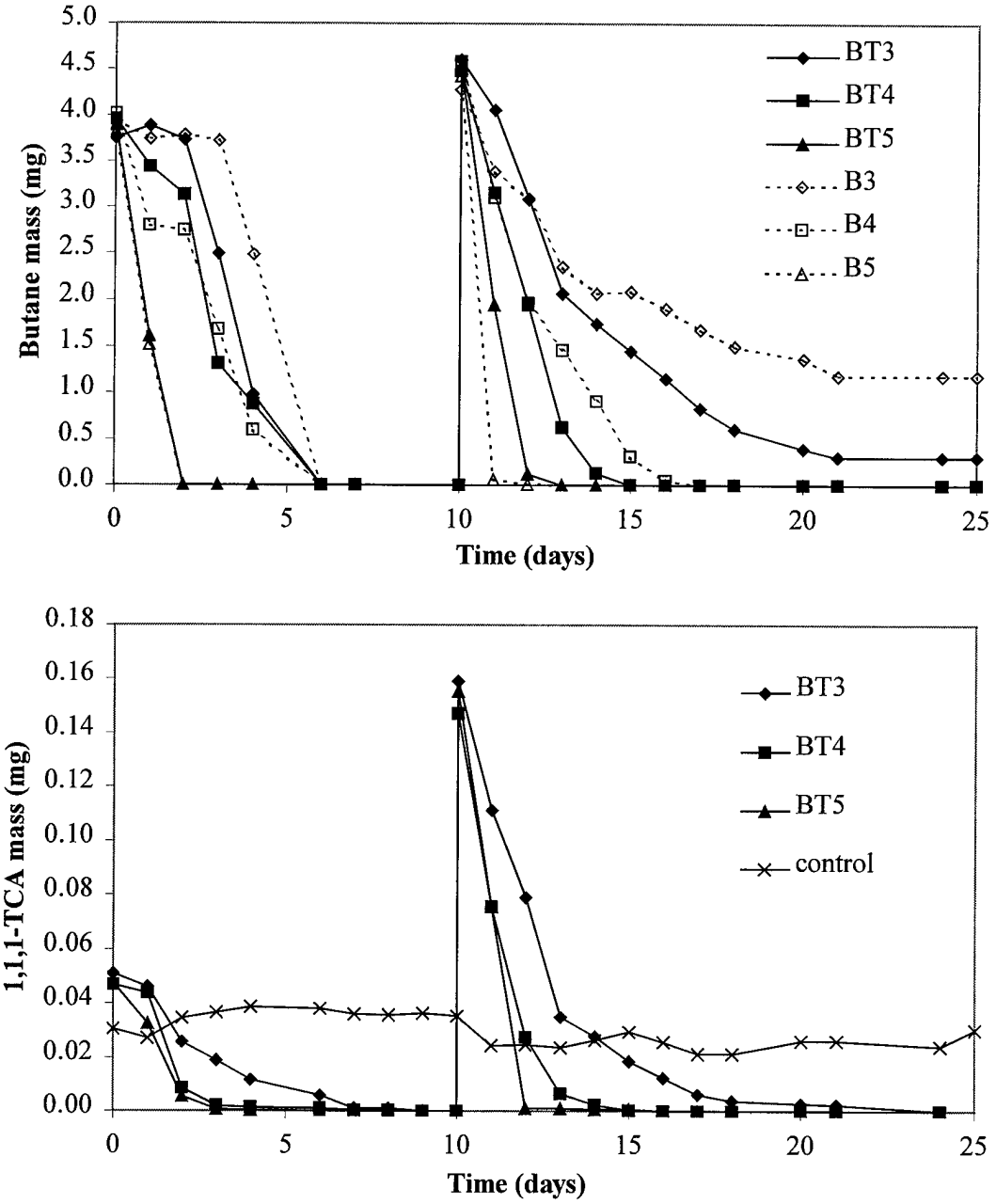


Figure 17 Bioaugmentation of a butane-utilizing mixed culture into microcosms with different nutrient amendments (see Table 5).

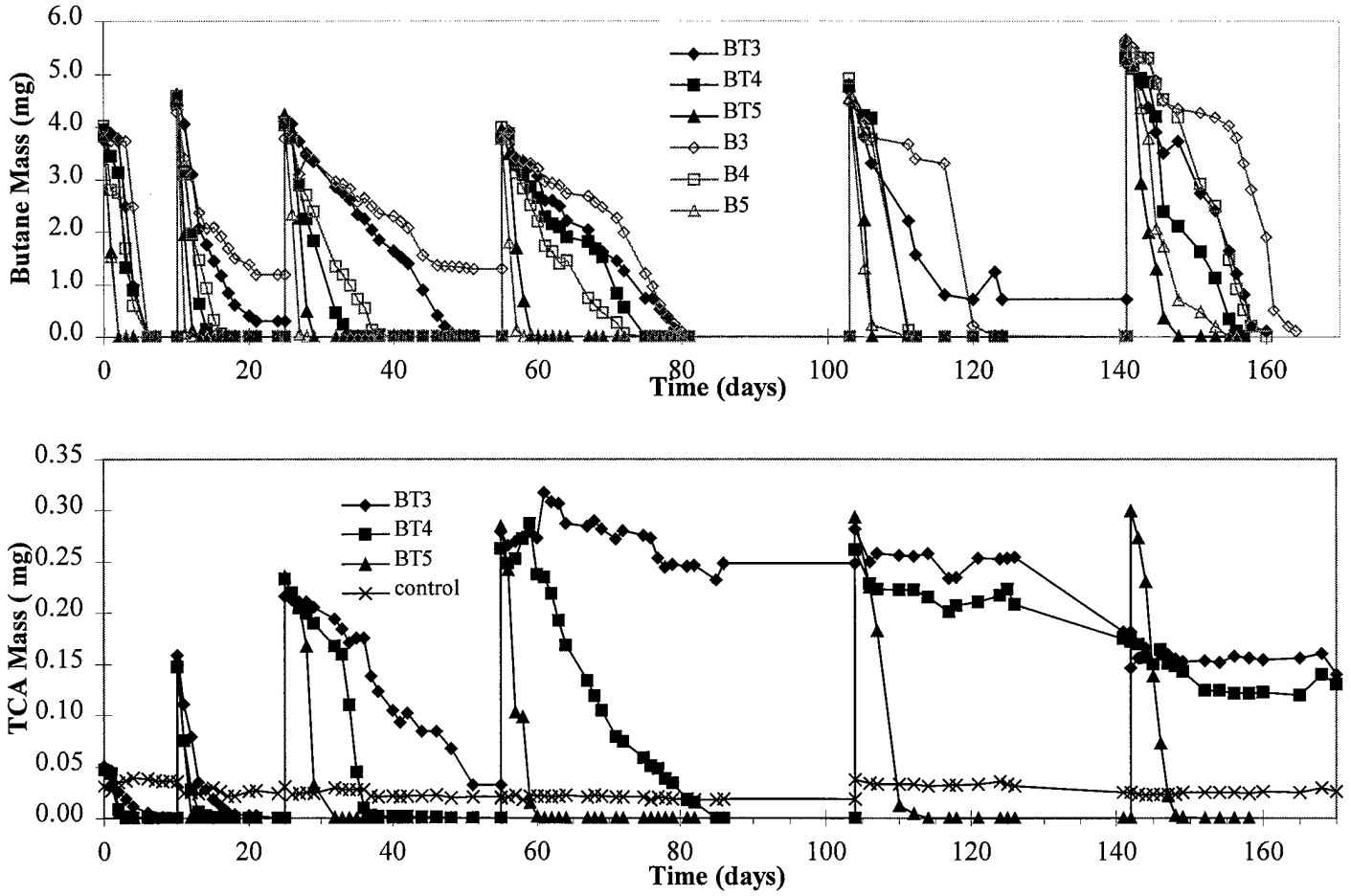


Figure 18 Bioaugmentation of butane-utilizing mixed culture with different nutrient amendments (see Table 5).

5. 1,1,1-TCA transformation efficiencies of the cultures growing in mineral media

After 150 days of stimulation, the butane-utilizers from microcosms B3, B4, B5, BT3, BT4 and BT5 were enriched in batch growth microcosm with mineral media. After 30 days of stimulation, 1,1,1-TCA transformation abilities were evaluated. Figure 19 showed butane utilization and 1,1,1-TCA transformation of enrichments obtained from bioaugmentation study (Figure 18) B3, B4, B5, BT3, BT4 and BT5. Enrichments from microcosms BT3 and BT4, that stopped transforming 1,1,1-TCA in soil microcosms, were able to transform 1,1,1-TCA. Total mass of 0.03 mg 1,1,1-TCA was completely transformed. All the microcosms transformed 1,1,1-TCA completely, including microcosms B3, B4 and B5, which were not previously exposed to 1,1,1-TCA. The results indicated that butane utilization efficiencies and 1,1,1-TCA transformation abilities depended on growth in mineral media. Past exposure to 1,1,1-TCA did not alter the transformation efficiencies of the enrichments.

6. DNA Fingerprinting.

DNA fingerprints from the microbes stimulated in the nutrient study (Figure 18) were carried out using primers HP7 (GCG AAG CCT AAC GCC) and GCA 12 (CGT GCC GAG CTG). Figures 20 and 21 show DNA fingerprints of the enriched cultures on days 80 and 160, respectively. The DNA fingerprints on day 80 and 160 were different, suggesting that the microbial populations changed between day 80 and 160. Microcosm B5, which showed no difference in butane degradation efficiency between 80 to 160 days, had similar DNA fingerprints at both times. This suggested that the microbial populations in microcosm B5, which had high mineral salt level, did not change significantly. Microcosm BT4, which degraded 1,1,1-TCA at 80 days but did not degrade 1,1,1-TCA at 160 days, showed distinctively different DNA fingerprints. The change in 1,1,1-TCA transformation efficiency might be a result from a shift in the population as indicated by the DNA fingerprints.

The DNA fingerprints between microcosms with different media amendments showed greater differences than microcosms that had the same amount of media, but with and without 1,1,1-TCA addition. The PCR results indicate that the exposure to 1,1,1-TCA did not affect the microbial population, as much as in the mineral media.

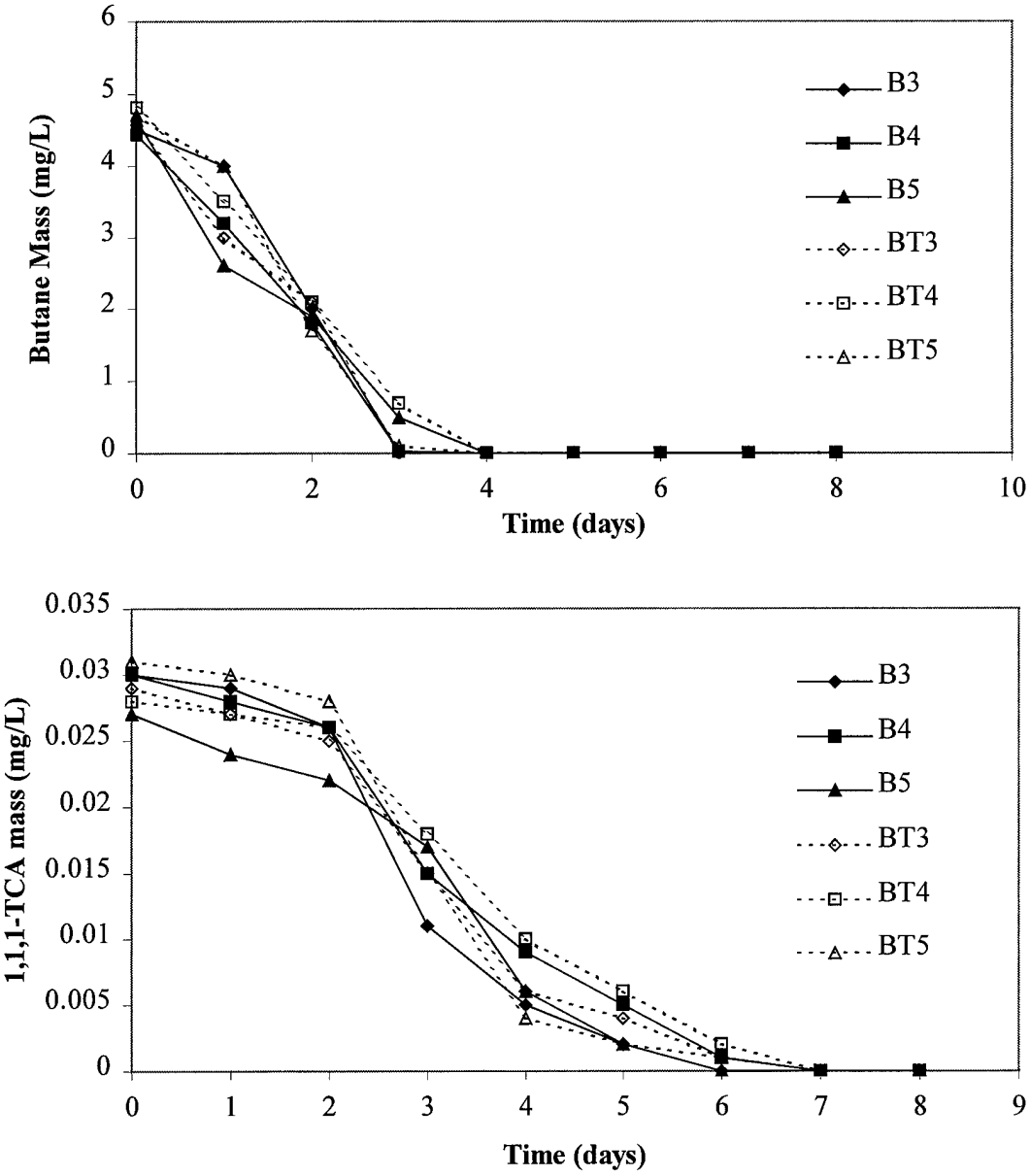


Figure 19 Butane utilization and 1,1,1-TCA transformation of microcosms inoculated with enrichments from B3, B4, B5, BT3, BT4 and BT5 grown in mineral salt media

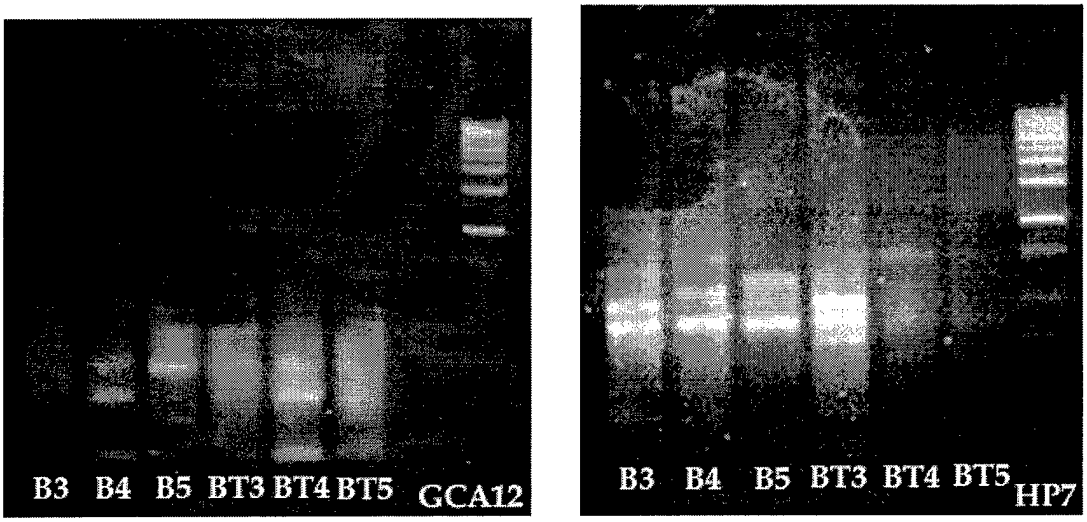


Figure 20 DNA fingerprints of microbial populations obtained from microcosms B3, B4, B5, BT3, BT4 and BT 5 on day 80.

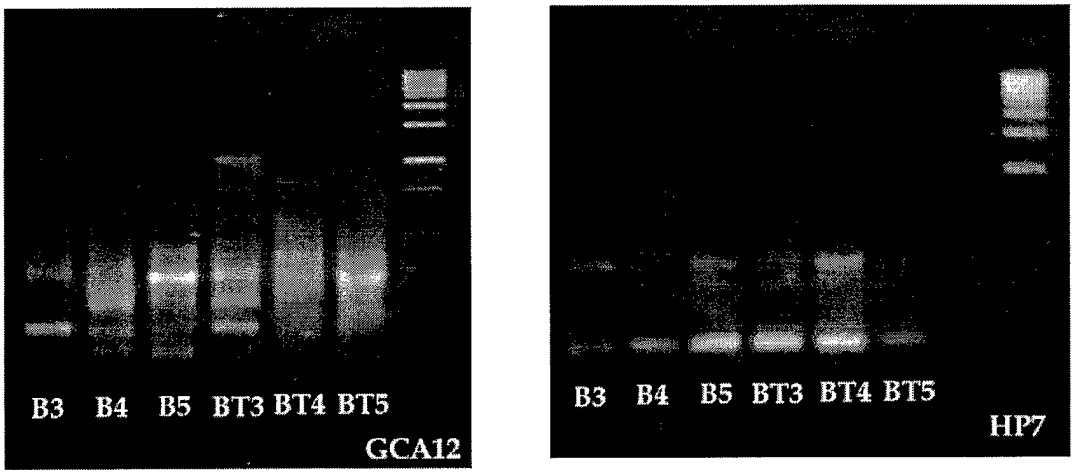


Figure 21 DNA fingerprints of microbial populations obtained from microcosms B3, B4, B5, BT3, BT4 and BT 5 on day 160.

7. Nutrient Studies with Propane-Utilizers

The propane-utilize used in bioaugmentation nutrient studies were enriched from McClellan AFB subsurface (Section IV). The set up for this experiment is presented in Table 5. Figure 22 shows propane utilization and 1,1,1-TCA transformation in the bioaugmentation study. Enrichments added to microcosms P4, PT4, P5, PT5 utilized propane completely. Microcosms PT4 and PT5 also transformed all of the 1,1,1-TCA. Microcosms P5 and PT5, which continued 50% mineral media had the highest propane degradation rates and PT5 had the fastest 1,1,1-TCA transformation rate. Microcosms P3 and PT3, which did not contain any mineral media, had the lowest propane degradation rate and only 33% of propane was transformed. In the second stimulation, microcosms P5 and PT5 completely utilized propane. All 1,1,1-TCA in PT5 was transformed. Only 45% and 33% of propane was transformed in microcosm P4 and PT4 respectively, and only 20% of 1,1,1-TCA was transformed in PT4. P3 and PT3 did not show any propane degradation, and 1,1,1-TCA in microcosm PT3 was not transformed.

The results from the nutrient studies with butane and propane-utilizers showed that both enrichments required mineral salt nutrient additions. 50% mineral salt additions were adequate for both augmented butane and propane-utilizers.

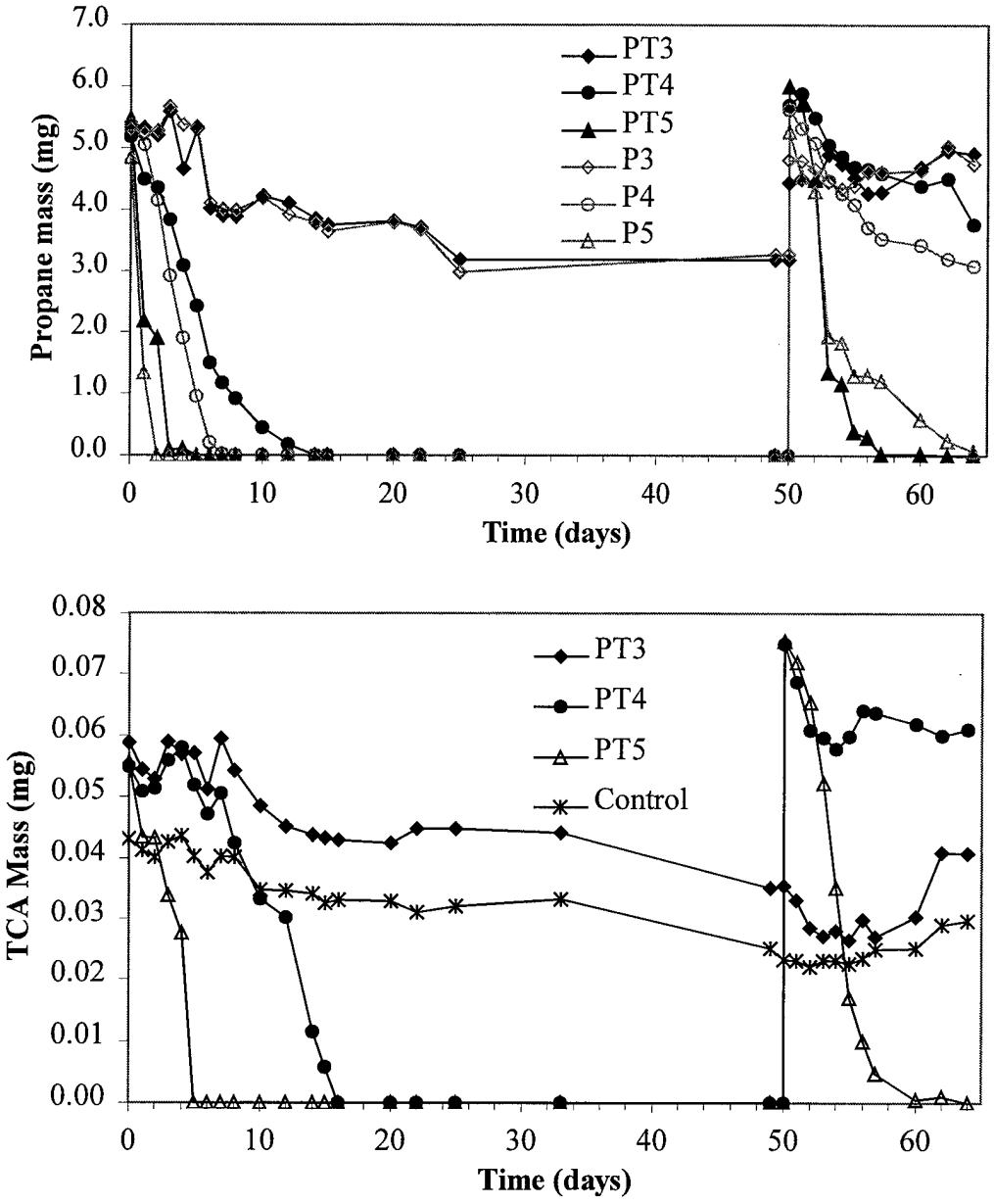


Figure 22 Bioaugmentation of propane-utilizing mixed culture into soil microcosms contain in differ nutrient amendments (see Table 5).

8. Enriching the butane and propane-utilizers from microcosms B1, B2, P1 and P2 for bioaugmentation

The long-term microcosm studied showed butane and propane-utilizers transformed 1,1,1-TCA well under the nutrient poor conditions of the groundwater. Thus bioaugmentation might be more successful if these strains were enriched and augmented under groundwater nutrient limited conditions. To test this hypothesis, microbial samples from microcosms B1, B2, P1, and P2 (Figure 8,9,13) were enriched in growth reactors containing mineral salt medium and butane and propane. After 30 days, the enrichments were bioaugmented into fresh soil groundwater microcosms without mineral media addition. The microcosms were fed with gaseous substrates and 1,1,1-TCA as in the previous studies. Figure 23 shows the butane utilization and 1,1,1-TCA transformation of the microcosms inoculated with enrichments from B1 and B2. Effective butane utilization and 1,1,1-TCA transformations were observed in both microcosms. Figure 24 shows the propane utilization and 1,1,1-TCA transformation in microcosms inoculated with enrichments from P1 and P2. Effective propane utilization and 1,1,1-TCA transformation were observed in both microcosms. In the forth stimulation, trichloroethylene (TCE) was added instead of 1,1,1-TCA. Both butane and propane-utilizers were able to transform TCE. The results showed that the inoculated strains, which were enriched in mineral salt medium for 30 days, had no difficulties utilizing the cometabolic substrates and transforming 1,1,1-TCA under groundwater conditions to minimal media added. These results when compared to those in Figure 17 and Figure 22, demonstrated the successful bioaugmentation with these strains, under the groundwater nutrient limited conditions. Therefore, butane or propane-utilizers, which are able to grow in nutrient-limited conditions, can be enriched in growth reactors for a short period of time to yield the high cell mass for bioaugmentation purposes without causing a shift to population that prefer nutrient rich conditions. The experiments were successfully repeated to assure that they were reproducible (data not shown).

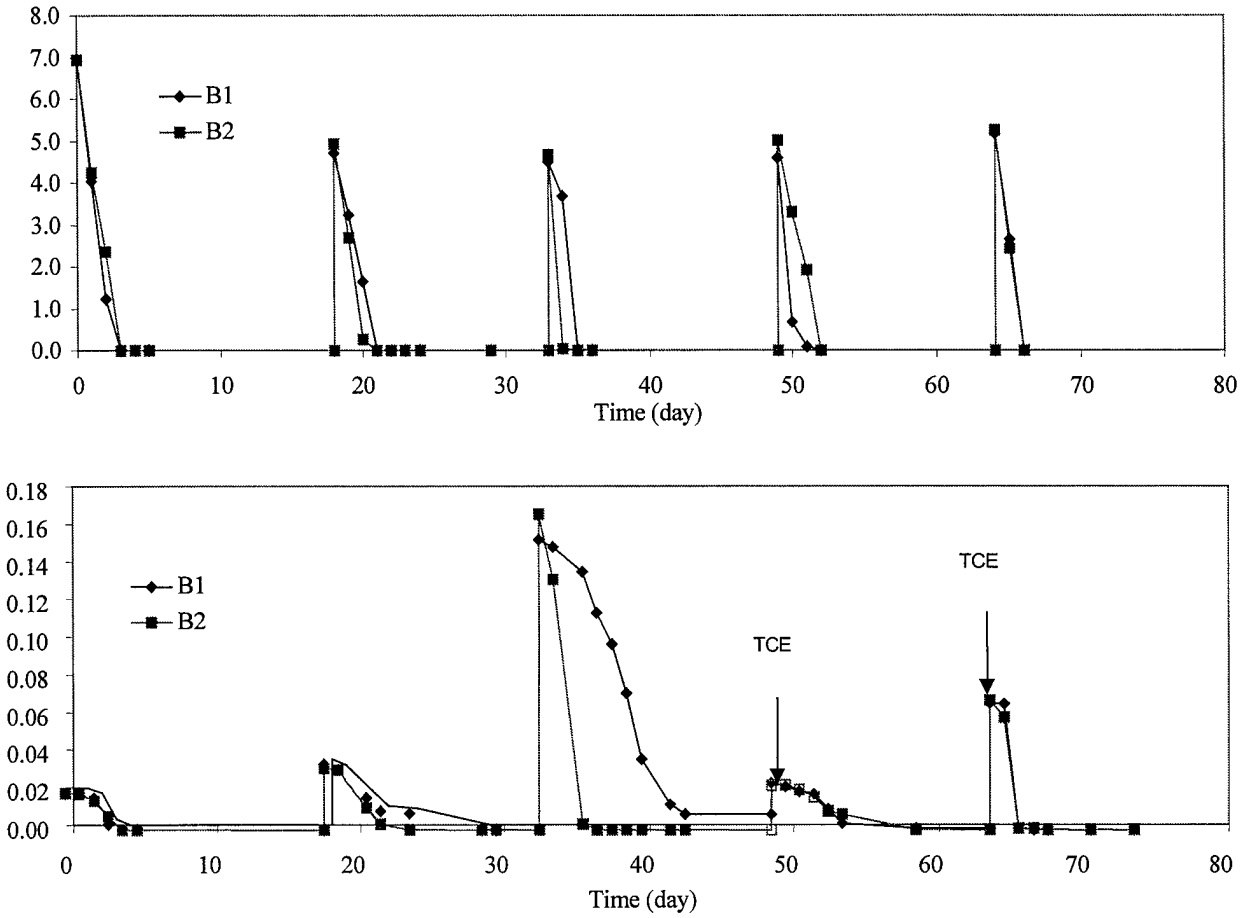


Figure 23 Butane utilization and 1,1,1-TCA transformation of groundwater microcosms inoculated with enrichments obtained from microcosms B1 and B2.

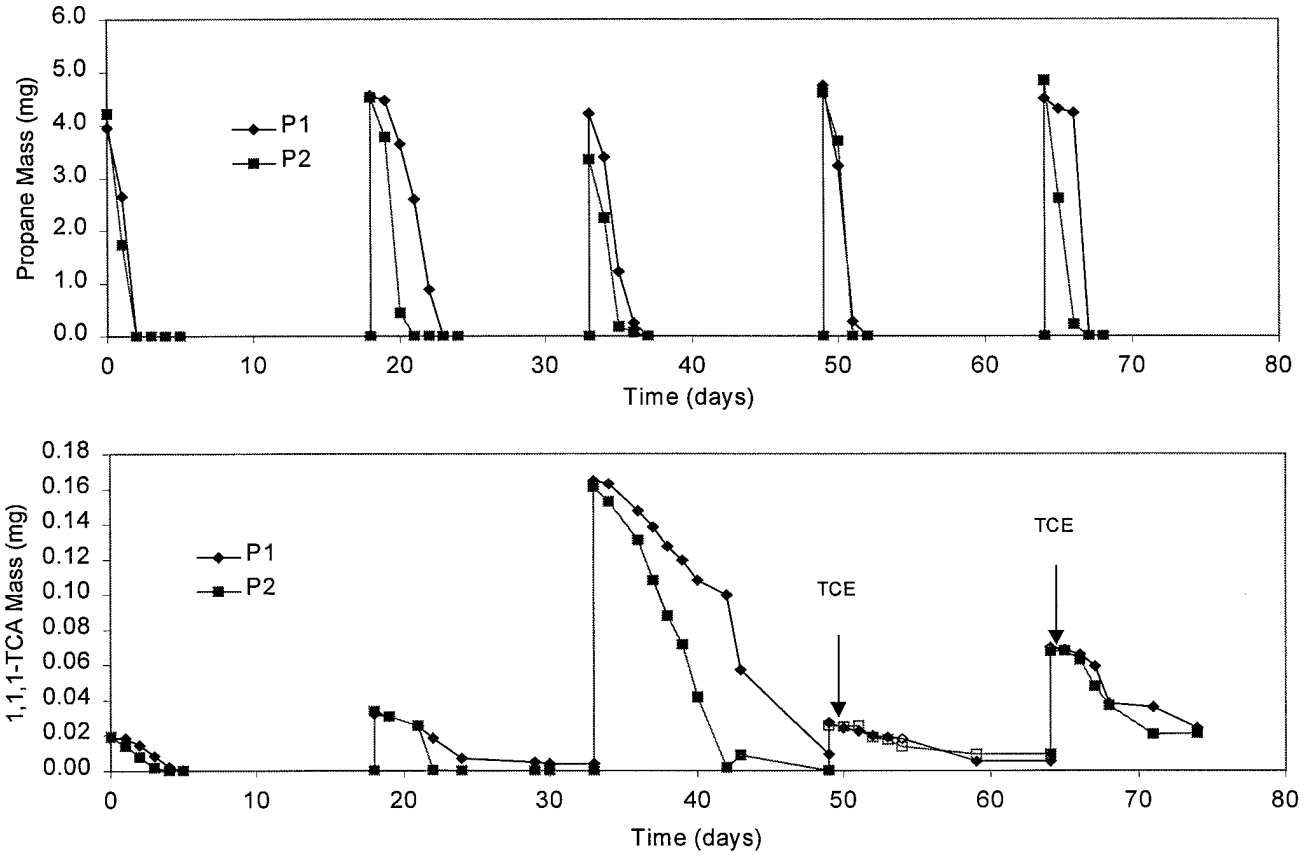


Figure 24 Propane utilization and 1,1,1-TCA transformation of groundwater microcosms inoculated with enrichments obtained from microcosms P1 and P2.

D. SUMMARY

The microcosm results indicate that indigenous butane and propane-utilizers could be stimulated in Moffett Field microcosms. Both butane and propane-utilizers that were stimulated remained active for more than a year after biostimulation without requiring of additional mineral supplements. However, there was a long lag period from 80 to 85 days before successful stimulation was achieved. Inoculating effective butane or propane-utilizers could reduce these lag periods to several days and would eliminate uncertainty about whether indigenous butane and propane-utilizers were actually present in the subsurface. Both butane and propane-utilizers showed satisfactory 1,1,1-TCA transformation. In a previous study (Kim et al., 1997) showed complete transformation of 1,1,1-TCA at concentrations as high as 2400 μg/L. In this study, the maximum 1,1,1-TCA concentration that was completely transformed was 8310 μg/L with the bioaugmented butane-utilizers (microcosm B2). The final transformation yields in butane and propane-utilizers are 0.04 and 0.07 mg 1,1,1-TCA/ mg substrate, respectively.

The bioaugmented butane-utilizers showed immediate 1,1,1-TCA cometabolism with high transformation efficiency. After prolong treatment and exposure to high 1,1,1-TCA concentrations, the transformation efficiency of bioaugmented butane-utilizers decreased, whereas the transformation efficiency in indigenous butane-utilizers improved. After 440 days, the transformation yields of bioaugmented and indigenous utilizers were the same. DNA fingerprints showed a strong similarity of the microbial populations at 440 days, suggesting that indigenous populations may be active in both microcosms. Unfortunately, DNA fingerprints earlier in the stimulation history were not available for comparison.

Bioaugmented and indigenous propane-utilizers showed similar transformation yields, but it is questionable whether bioaugmentation actually reduced the lag period. The transformation yields increased after prolonged incubation and treatment and were similar in both microcosms. DNA fingerprints showed similarities in microbial population between indigenous and bioaugmented propane-utilizers. The results may indicate that indigenous strains were predominant at 440 days. No evidence is available to indicate whether bioaugmented propane-utilizers were stimulated in microcosm P2 at early time. The results tend to suggest that indigenous strains were stimulated in both microcosms. Due to the long lag period experienced upon the initial stimulation of approximately 80 days, we can not rule out the possibility that the "indigenous strains" were actually introduced to the microcosms during the sampling. Several precautions would help future studies. A radiated control, fed the growth substrate, should be included in the experimental set-up. This might demonstrate the introduction of microorganisms during microcosm operation. Also microcosms should be constructed and fed growth substrate, but are not sampled until there is evidence of substrate uptake in other microcosms. If these microcosms also show uptake of substrate, it would help rule out the possibility of microbial contamination.

After 440 days of repeated stimulation, the propane-utilizers showed higher 1,1,1-TCA transformation yields than butane-utilizers and also displayed more stability. Bioaugmented

butane-utilizers, although immediately effective, became unstable when exposed to high 1,1,1-TCA concentrations and prolonged treatment.

Enriching cultures in growth reactors that contained high mineral salt supplement can result in a shift in the microbial population. The enrichments that were grown in the growth reactor for long periods (150 to 210 days) had trouble adapting to aquifer groundwater conditions upon inoculation. Mineral salt supplements had to be added into the soil groundwater microcosms to assure the effective substrate utilization and 1,1,1-TCA transformation of inoculated enrichments. However, the enrichments that performed well under nutrient poor condition when grown in growth reactors for only a short period of time (30days), did not have difficulties adapting to groundwater conditions once reinoculated.

E. CONCLUSIONS

1. Indigenous butane and propane-utilizing microorganisms could be stimulated from Moffett Field's groundwater and aquifer materials. Both indigenous butane and propane-utilizers were capable of transforming 1,1,1-TCA.

2. Butane and propane-utilizers stimulated from Moffett Field's groundwater could actively transformed 1,1,1-TCA for more than one year with repeated biostimulation without requiring additional mineral nutrient supplements.

3. Inoculating butane or propane-utilizers grown in laboratory could reduce long lag periods. Since indigenous strains performed well under groundwater nutrient limited conditions, one should consider reinoculating indigenous strains before using strains from other sites

4. Bioaugmented butane-utilizers showed better 1,1,1-TCA cometabolism initially. After prolonged treatments, the transformation yields of bioaugmented butane-utilizers decreased, whereas that of indigenous butane-utilizers increased. Ultimately, both enrichments showed similar transformation yields. DNA fingerprints indicated that at the end of the study, the populations in both microcosms were very similar, suggesting that indigenous populations had become dominant in the microcosms.

5. Indigenous and bioaugmented propane-utilizers showed similar transformation yields throughout the study. However, DNA fingerprints showed that the microbial populations in both microcosms were similar. Indigenous utilizers may have predominated in both microcosms through the experiments.

6. 1,1,1-TCA transformation appears to be competitively inhibited by growth substrates (butane or propane).

7. The exposure of 1,1,1-TCA up to 0.3 mg (3.51 mg/L aqueous concentration) did not effect the enrichments' ability to transform 1,1,1-TCA.

8. Propane-utilizers are better candidates for bioaugmentation since the final transformation yields were higher than butane-utilizers, and propane-utilizers were more stable with repeated stimulation than butane-utilizers. However, bioaugmentation with indigenous strains should be considered to reduce the long lag time.

9. Growing the enrichments in mineral salt mediums for too long can cause a shift in population that prefers high nutrient conditions. This reduces the efficiency upon inoculated the enrichments into groundwater nutrient limited conditions. The media along with bioaugmentation can help maintain the efficiency. However, it would be better to find strains that do not need nutrient supplements.

10. Indigenous microbial populations that are able to grow in nutrient-limited conditions and effectively degrade 1,1,1-TCA, are best choices for the bioaugmentation. We were successful in enriching these cultures in mineral salt medium for a short period of time to yield high cell mass, and then bioaugmenting these into nutrient limited groundwater to achieve short lag periods and effective 1,1,1-TCA transformation.

F. ENGINEERING SIGNIFICANCE

The butane and propane-utilizers in Moffett Field were capable of transforming 1,1,1-TCA with satisfying transformation yields and efficiencies. The strains remained effective for over a year with repeated biostimulation. Both indigenous butane and propane-utilizers took more than 80 days to be stimulated, which is impractical for a field demonstration. Also with such long lag periods for aerobic microorganisms, one can not rule out the possibility of laboratory contamination. Thus one can not be certain that the stimulated strains are actually present in the subsurface. Thus bioaugmentation will likely be required to insure success in the field.

The bioaugmentation study demonstrated that inoculating laboratory grown butane or propane-utilizers, that were capable of transforming 1,1,1-TCA, could significantly reduce the long lag period. However, after repeated stimulation and prolonged treatments, DNA fingerprints indicated similar population existed in the microcosm. The results may indicate that augmented strains, although more effective in the beginning, were out-competed by the indigenous strains. Bioaugmentation with indigenous strains will reduce the concerns regarding the strains' adjustment to the environmental conditions.

The limitation of stimulating indigenous butane and propane-utilizers in the field is the lag time. Bioaugmentation reduced the lag period to several days, but the survival of the augmented microbes in the new environment was uncertain. The indigenous strains can be

enriched and used as inoculum, although the transformation yields may be lower than that of other enrichments. Reinoculating indigenous strains would reduce the concerns regarding microbial ecology, such as interspecies competition and assure a better adapting of microbes to nutrient conditions in the groundwater.

The indigenous strains can be grown in soil microcosms and enriched in mineral salt nutrients prior to bioaugmentation. The results indicated that enriching the strains in mineral salt medium for extended periods appeared to select for strains that rely on abundant mineral salt medium. Therefore, when the enrichments were reinoculated into the nutrient limited conditions, their abilities to degrade substrates and transform 1,1,1-TCA was limited. The nutrient study showed that mineral salt nutrient supplements can be added along with substrates to provide favorable conditions for the bioaugmentation. Nutrient addition of 5% was proven to be inadequate for the long-term treatment, 50% media addition was sufficient to maintain the activity, but would be impractical for a field study.

Inoculating the strains that were capable of cometabolizing 1,1,1-TCA under nutrient limited conditions appeared to be more promising. The enrichments, which grew on butane and propane in Moffett Field microcosms, performed well under nutrient limited conditions. Indigenous strains were effective over extended periods, especially propane-utilizers, which were more consistent than butane-utilizers. Accordingly, these indigenous strains were highly suitable for use as inoculums to help in stimulating 1,1,1-TCA cometabolism in Moffett Field conditions. Our final results showed these strains could be inoculated for effective bioremediation.

At some sites, such as McClellan Air Force Base, indigenous butane-utilizers were unable to transform 1,1,1-TCA. Therefore, enrichments obtained from other sites could be inoculated for use. Since the characteristics of the indigenous strains and augmented strains way differ, the interspecies competition and nutrient conditions are of concern. The indigenous microorganisms may become predominant, therefore, augmented enrichments may not be successful for long term bioremediation. The augmented strains may not be able to adapt to the nutrient conditions at the new site. More research is needed to determine a conditions under which successful bioaugmentation might be achieved in this case.

SECTION IV

COMPARISON OF LONG-TERM TCE TRANSFORMATION ABILITY OF METHANE AND PROPANE-UTILIZING MICROORGANISMS STIMULATED FROM MCCLELLAN AFB SUBSURFACE

A. INTRODUCTION

Trichloroethylene (TCE) is one of the most widespread contaminants in soil and groundwater, due to its use as a degreaser, dry cleaning solvent, and extraction agent in industry and government facilities including military installations (Imfante and Tsongas, 1982; Westrick et al., 1984). Many military installations including McClellan Air Force Base practiced long-term land disposal of TCE throughout the 1970s. TCE concentrations greater than 0.5 mg TCE/L have been detected in groundwater at McClellan AFB. Previous studies have demonstrated that in situ bioremediation using the subsurface as a bioreactor to eliminate above ground treatment has good potential for remediating chlorinated aliphatic hydrocarbon (CAH) contamination (Semprini et al., 1992. This technology may be capable of minimizing remediation costs and may reduce the time required for restoring contaminated aquifers.

CAHs, including TCE, can be degraded cometabolically into nontoxic end products by many types of aerobic microorganisms. Aerobic microorganisms grown on methane, propane, ammonia, toluene, and phenol can initiate CAH cometabolism (McCarty and Semprini, 1993). Much research has focused on TCE cometabolism by methane-utilizing mixed and pure cultures (Alvarez-Cohen and McCarty, 1991a and 1991b; Fogel et al., 1986; Little et al 1988; Henson et al., 1988; Oldenhuis et al., 1989; Tsien et al 1989). In contrast, there are only a few investigations of CAH transformation by propane (Wackett et al., 1989; Keenan et al., 1993; Wilcox et al., 1995) and butane-utilizing bacteria (Kim et al., 1997).

Aerobic TCE cometabolism has revealed inhibitory effects limiting TCE transformation. TCE oxidation requires the expression of an oxygenase enzyme and a source of reductant (e.g., NADH). The loss of enzyme activity and/or reductant supply significantly reduces the capacity to transform TCE (Chang and Alvarez-Cohen, 1995, 1996). Furthermore, competitive inhibition between TCE and the substrate sites decreases the TCE transformation rate (Broholm et al., 1990; Oldenhuis et al., 1991; Semprini et al., 1991). TCE product toxicity also inhibits TCE transformation (Alvarez-Cohen and McCarty, 1991; Oldenhuis et al., 1991).

Methane-utilizers can maintain TCE transformation ability for a limited time after methane is consumed by regenerating a source of reducing energy (NADH) using formate and methanol. Alternative energy sources such as formate and methanol, which are methane catabolic intermediates, temporally enhance TCE transformation (Oldenhuis et al., 1989; Alvarez-Cohen and McCarty, 1991; Semprini et al., 1991; Janssen et al., 1988). Methane-utilizers can also use poly-β-hydroxybutyrate (PHB) as an endogenous energy source to regenerate NADH during TCE transformation (Asenjo and Suk, 1986; Henrysson and McCarty, 1993). PHB is an intracellular reserve polymer whose synthesis serves as an

electron sink for microorganisms under growth-limited conditions. The intracellular reducing equivalent improves the extended TCE transformation throughout catabolism of stored PHB (Henrysson and McCarty, 1993; Henry and Grbic-Galic, 1991).

Among the gaseous alkanes, most of the research has focused on microorganisms that grow on methane. However, biomass production rates from methane are limited by methane mass transfer because the solubility of methane is relatively low. Therefore, normal alkanes with higher transfer rates and solubility limits such as propane and *n*-butane have been used for higher biomass production. Biomass yields with propane and butane are approximately 1.4 times higher than with methane (McLee et al., 1972). Propane and butane are cheap, readily available substrates that are nontoxic and not regulated. Thus, it is possible to obtain regulatory approval to use these compounds for enhanced in situ bioremediation.

Previous studies have indicated that long-term in situ bioremediation might be difficult due to the microorganisms' inability to continue TCE degradation for extended times. Transformation product toxicity can have an effect on the basis of TCE transformation ability. Fields studies have evaluated TCE transformation potential for periods of months (Roberts et al., 1989; Semprini et al., 1990, 1991, 1995; McCarty, 1993; Hopkins et al., 1993). The column microcosm studies with indigenous microorganisms grown on phenol showed some loss in TCE transformation ability after 280 days (Munakata et al., 1997). However, Jenal-Wanner and McCarty (1997) have reported no loss of TCE transformation ability over a 1-year study period of semicontinuous slurry microcosms stimulated by phenol and toluene on uncontaminated soil from Moffett AFB. In this section, we presented data on TCE transformation in long-term batch-incubated microcosms by indigenous methane- and propane-utilizing microorganisms stimulated by subsurface aquifer solids and groundwater from McClellan AFB in Sacramento, California. We studied the ability to maintain long-term TCE transformation as TCE concentrations gradually increased over a 1-year period. Propane-utilizers continued to transform TCE for up to 30 days after propane was consumed in the microcosms.

B. MATERIALS AND METHODS

1. Long-term batch microcosm studies with aquifer solids

The microcosms were constructed using aquifer solids and groundwater from McClellan AFB. Methane, propane, and butane were used as cometabolic growth substrates. The microcosm method was adapted from Broholm et al. (1990) and Yi Mu and Scow (1994). Duplicate microcosms were prepared for each substrate tested. The microcosms were constructed using 125-ml amber serum bottles (Wheaton Class Co., Millville, NJ.). Aquifer solids were wet sieved with site groundwater under a laminar flowhood using a No. 8 sieve (2.38-mm opening) to remove large particles. The site groundwater was filtered (50 mL) through a sterilized 0.45-μm filter before use. Wet solids of filtered groundwater were added to each microcosm, leaving a 60-mL air-filled headspace as a source of oxygen. The headspace permitted the sampling of the gaseous substrate, oxygen, and TCE. The microcosms were crimp-sealed with a Teflon™ butyl rubber cap (Kimble Co., IL), then inverted and incubated at room temperature on a shaker table (G-10 Gyrotry Shaker, Newbrunhwick Scientific) at 120 rpm. The microcosms were maintained

for 1 year, with periodic sampling and groundwater exchanges with readdition of growth substrates and TCE.

Control microcosms included (1) TCE controls containing aquifer solids, groundwater, and TCE, but lacking the growth substrate; (2) sterilized controls prepared in the above manner, but exposed for 11 hours to a cobalt-60 gamma irradiation source. After irradiation, filtered groundwater (0.45 μm) was added under laminar flowhood. The addition of the 0.45-μm of filtered groundwater potentially resulted in a source of microorganisms to these controls.

Groundwater (25 mL) was exchanged in the microcosms prior to additions of the growth substrate and TCE. The groundwater was amended with nitrate (30 mg/L), because nitrogen was found to be limiting in the groundwater. Prior to exchanging the groundwater, the microcosms were centrifuged for 20 min at 2000 rpm to keep the microorganisms in the microcosms. The serum caps were then removed under laminar flowhood, groundwater was replaced, and the microcosms were resealed. With each exchange, the mass of TCE added was increased, while maintaining a constant mass addition of growth substrate.

2. Chemicals

Trichloroethylene (TCE; >99%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Methane (>99.9%) was purchased from Airco (Vancouver, WA). Propane (10 % in nitrogen) and butane (10% in nitrogen) were obtained from Aldrich Chemical Co. (Milwaukee, WI). A saturated TCE stock solution was prepared by adding 4 mL of pure TCE in a 125-mL capped serum bottle. The bottle was shaken and allowed to settle for at least 24 hours before use. Methane, propane, and butane were transferred from gas containers to batch microcosms by direct volume additions with gastight syringes (Hamilton Co., Reno, NV).

3. Analytical methods

Methane, propane, butane, TCE, and oxygen were measured in headspace samples of the microcosms. TCE concentrations were measured with a Hewlett Packard (Wilmington, DE) 5890 gas chromatograph equipped with a ^{63}Ni electron capture detector. Separation was obtained by using a stainless steel packed column (1/8" x 8'; 15% squalene; CPAW-DMCS; 80/100; 5327PC, Alltech, Deerfield, IL) operated isothermally at 80°C. An argon/methane (95/5) mixture at head pressure of 60 psi was used as the carrier gas. A 100-μl headspace sample was analyzed. The method was calibrated using external standards.

TCE aqueous concentrations were quantified by purge and trap using a modified version of standard U.S. Environmental Protection Agency (EPA) Method 8010. A Hewlett Packard Purge and Trap model 7695 was used in conjunction with a Hewlett Packard 5890 gas chromatograph equipped with a Hall conductivity detector. A 100-μL sample was diluted in 5 mL of distilled water and then transferred into the trap of the purge and trap unit. Separations were obtained by using a capillary column (HP-624; 19091v-433; 1.4 μm; 30 m length; Hewlett Packard, Wilmington, DE) operated with a temperature gradient.

Headspace oxygen concentrations were determined on a Fisher Model 25V gas partitioner using nitrogen as the carrier gas. A 100-μL headspace sample was obtained with a Pressure-Lok gastight syringe (Hamilton Co., Reno, NV). Separations were obtained by using a stainless steel packed column (Supelco, I., Bellefonte, PA). Oxygen gas standard was used for calibration.

Methane, propane, and butane concentrations were quantified by headspace analysis using a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector coupled with a 1.0-m Hayesep Q stainless steel micropacked column (Restek Corporation, Bellefonte, PA). A 100-μL sample was used. The method was calibrated using external standards.

Nitrate concentrations were determined on a Dionex 4000I ion chromatograph. A Dionex Ionpac AS4A column, which utilizes a regenerant containing an H_2SO_4, Na_2CO_3, and $NaHCO_3$ eluent composition, was used for the chromatography separation. A 50-μL aqueous sample was analyzed. The method was calibrated using external standards.

C. RESULTS

1. The evaluation of indigenous microbial activity in the McClellan AFB subsurface cores

The initial microcosm studies were performed to determine whether indigenous microbial activity in the McClellan AFB subsurface utilized the different gaseous cometabolic substrates. Prior to TCE addition, methane, propane, and butane microcosms were fed successively to the microcosm. Table 11 presents the mass histories of growth substrate addition and the lag time for substrate utilization during the initial incubation of the microcosms. The results demonstrate that a diverse microbial community exists in the McClellan AFB subsurface. Microbes could be stimulated on all of the substrates tested. Stimulation of methane-utilizers in active microcosms was most rapid. The lag time in propane and butane microcosms was similar and about twice that observed for methane-utilizers. Complete removal of the substrate was observed within 2 to 3 days in the active microcosms after the initial lag periods. Oxygen uptake in each batch microcosm correlated well with substrate utilization.

Table 11. Lag time for growth substrate utilization during the initial microcosm incubation and maximum TCE transformation yields achieved with the different substrates.

Growth substrate	Microcosms	Mass of substrate added (mg)	Lag time before substrate utilization (days)	Average time required for substrate utilization (days)	Maximum transformation yields for TCE (g TCE/g substrate)
Methane	M#1	4.0	10	2	0.060
	M#2	4.5	10	2	0.048
	M#3 (Control)	4.5	55	5	0.068
Propane	P#1	4.0	24	2	0.028
	P#2	5.0	25	2	0.023
	P#3 (Control)	5.5	50	2	0.048
Butane	B#1	4.5	20	3	0
	B#2	5.5	20	3	0
	B#3 (Control)	5.7	45	15	0

The uptake of growth substrate was eventually observed in the sterilized controls with lag times ranging from about 45 to 55 days for methane, propane, and butane. The noncontrols were stimulated with much shorter lag periods than the sterilized control microcosms. The presence of microorganisms in all sterilized control microcosms may have resulted from using 0.45 μm of filtered groundwater. Experiments were continued with the controls to compare TCE transformation with the active microcosms. TCE addition was started after five additions of growth substrate. The transformation yields for TCE reported in Table 11 are the maximum observed with increases in TCE concentration with successive readditions of TCE and the growth substrate. Methane- and propane-utilizing microorganisms enriched from the site were active in promoting TCE cometabolism, whereas the butane-utilizers exhibited no ability to transform TCE. The maximum transformation yields of 0.068 g TCE/g methane and 0.048 g TCE/g propane were observed on the methane-utilizers (M#3) and propane-utilizers (P#3), respectively, demonstrating that the stimulated controls exhibited the highest transformation yields.

2. Long-term batch microcosm studies and the effect of TCE concentration

Long-term batch microcosm tests were conducted to study the effects of increasing of TCE concentrations on the rates and extents of TCE transformation, and the microorganism's ability to cope with increasing TCE concentrations. The aqueous TCE concentration was increased gradually from 0 to 7000 μg TCE/L (500 μg TCE) over a 1-year period, while maintaining a constant mass of substrate addition. The maximum sustainable ratio of TCE transformed to substrate consumed was determined.

Figures 25 and 26 shows the mass histories of methane and propane, respectively relative to TCE over a 1-year period. TCE additions were performed after several initial additions of growth substrate. Methane- and propane-utilizers were active toward TCE cometabolism and the zero-order rates of TCE transformations increased in all methane and propane microcosms at low TCE concentrations. Higher rates of TCE transformation were associated with the highest rates of substrate consumption. In both of methane and propane microcosms, the rate and the extent of TCE transformations varied among microcosms and correlated well with the rate of primary substrate utilization. The increase in TCE concentration resulted in different TCE transformation activities. The microcosms showed differences in TCE transformation yields developing with time. All microcosms remained active toward primary substrate utilization over 1-year period.

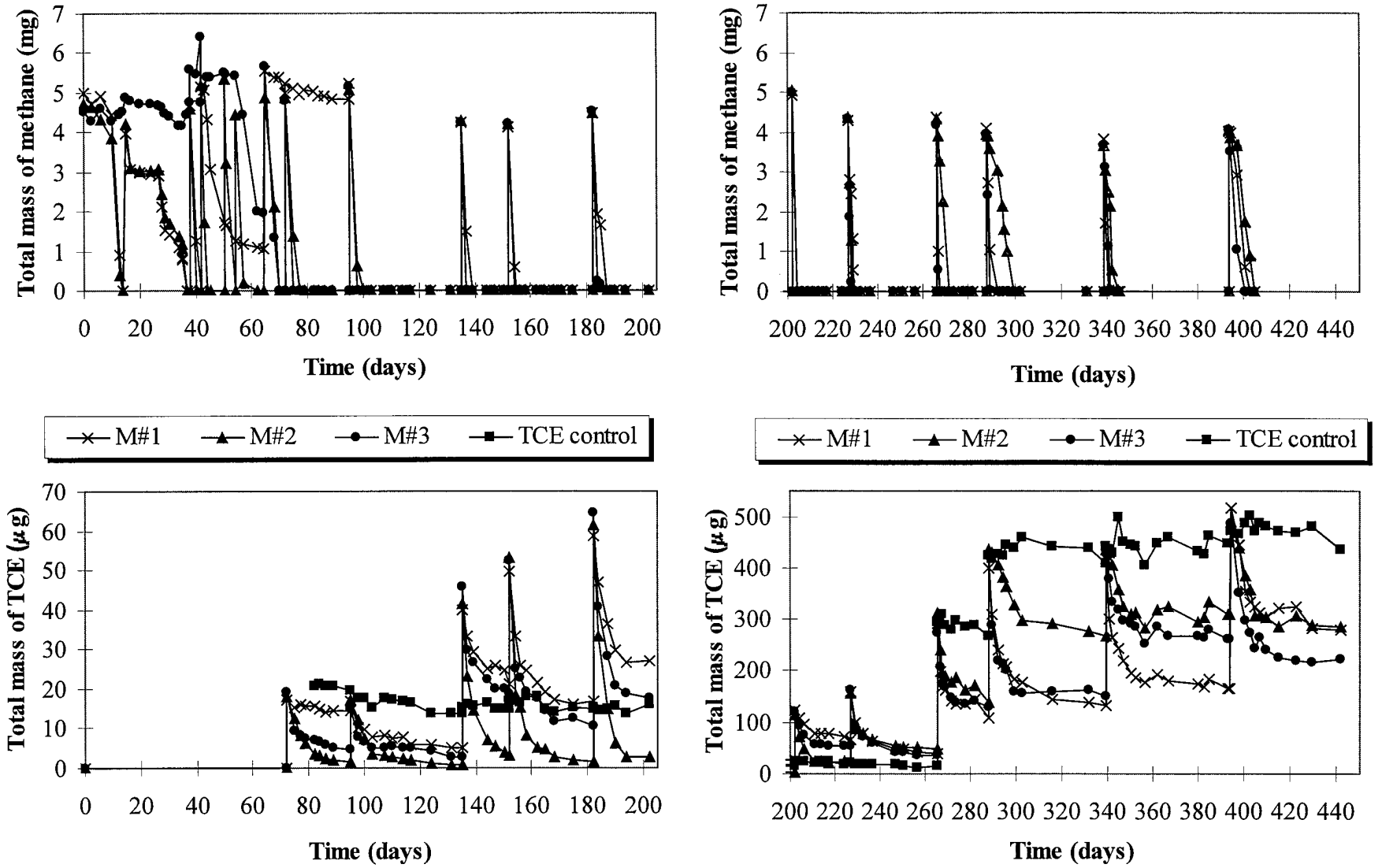


Figure 25. Mass histories of methane and TCE with increasing TCE concentration. Symbols: x, M#1 (TCE and methane); ▲, M#2 (TCE and methane); •, M#3 (TCE and methane); ■, TCE control (TCE without methane). The TCE mass from 0 to 500 µg corresponds to an aqueous TCE concentration from 0 to 7000 µg/L.

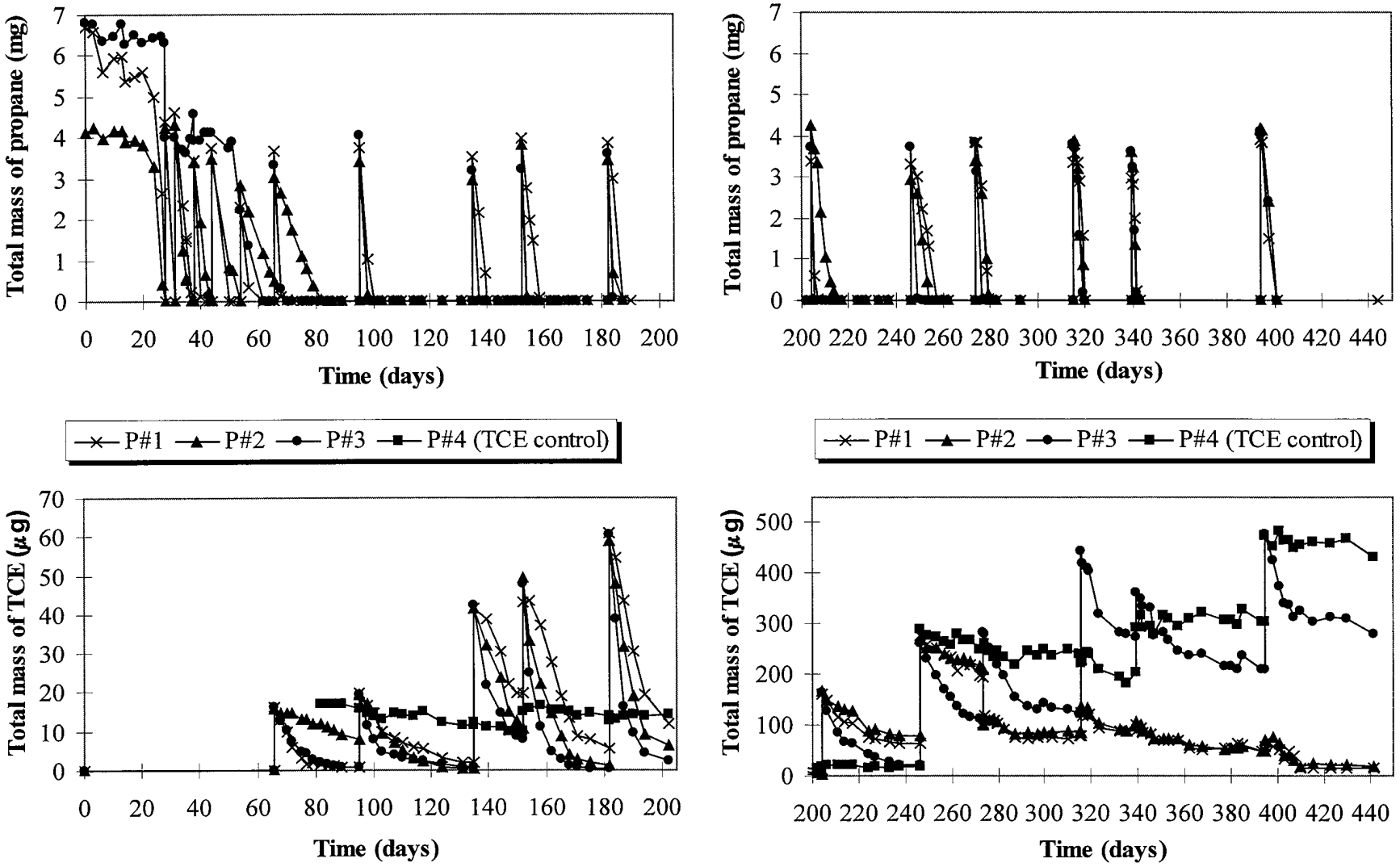


Figure 26. Mass histories of propane and TCE with increasing TCE concentration. Symbols: x, P#1 (TCE and propane); ▲, P#2 (TCE and propane); •, P#3 (TCE and propane); ■, TCE control (TCE without propane). The TCE mass from 0 to 500 µg corresponds to an aqueous TCE concentration from 0 to 7000 µg/L.

3. The prolonged TCE activity of methane and propane utilizers after consumption of substrate

The methane-utilizers and propane-utilizers showed different abilities to remain active toward TCE cometabolism after the primary substrate was consumed. Figures 27 and 28 illustrate TCE transformation abilities on the methane-utilizers compared to the propane-utilizers and present the logarithm of TCE mass versus time. The rates of TCE transformation are correlated with the rates of methane utilization. Higher rates of TCE transformation were observed when methane was being consumed. The rate of TCE transformation decreased soon after methane was depleted and followed a first-order rate of removal until transformation ceased. However, TCE transformation continued for a period of about 10 days after methane was consumed. This prolonged activity was very reproducible in the successive readditions of methane and TCE. The propane microcosms (Figure 28) follow first-order transformation kinetics for up to 20 days after propane was consumed. The rates of TCE transformation were correlated with the rates of propane utilization. The first-order TCE transformation and correlation with rates was very reproducible upon successive readditions to the microcosms.

Table 12 shows the averaged first-order rate coefficient and the average period of measurable TCE activity for three methane and three propane-utilizers both before and after the primary substrate was consumed. The methane-utilizers transformed TCE at a much faster rate than the propane-utilizers in the presence of the primary substrate. Initial first-order rates for methane ranged from 0.14 to 0.21 day $^{-1}$, compared to rates of 0.09 to 0.010 day $^{-1}$ for propane when primary substrate was being consumed. The rates of primary substrate utilization were similar for the methane- and propane-utilizers. The average values were estimated from individual estimates from successive additions of TCE and growth substrate over the 1-year study period. All methane cultures showed an ability to transform TCE for a period of about 9 days after methane was consumed with the averaged first-order kinetic rates ranging from about 0.04 day $^{-1}$ to 0.08 day $^{-1}$. The propane cultures remained active for longer periods (up to 20 days) and had higher first-order rate coefficients after the primary substrate was consumed. Their averaged first-order kinetic rate ranged from about 0.09 day^{-1} to 0.102 day^{-1}. Propane microcosm (P#3) remained active for the longest period of TCE transformation (about 23 days) after propane was consumed. It is interesting that the average first-order rates are similar for all propane microcosms. Microcosm P#3, however, was stimulated from the radiated control and this culture may have resulted from microbes in groundwater that passed through the 0.45-μm filter.

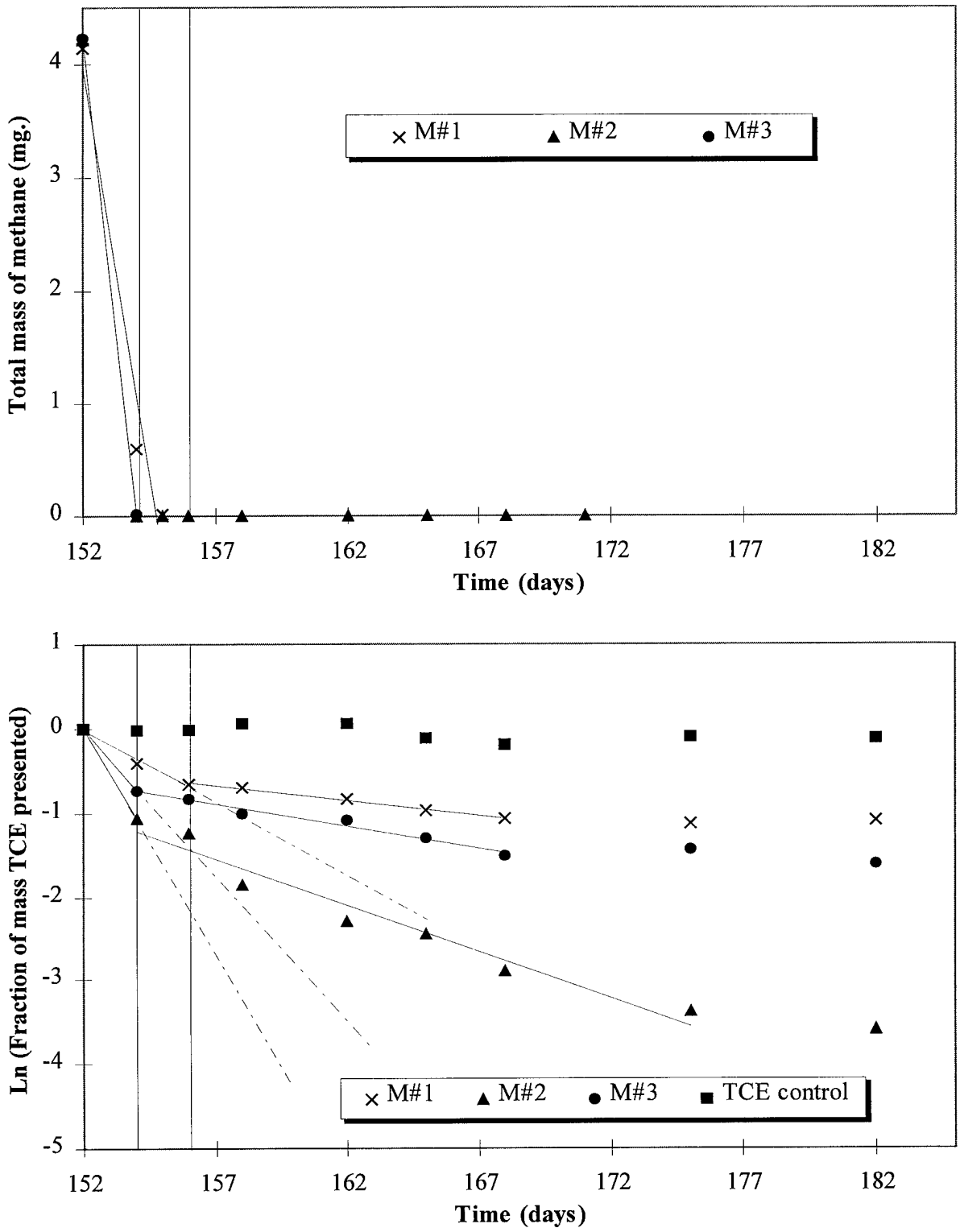


Figure 27. First order rate of TCE transformation and mass histories of methane consumption versus time. Symbols: x, M#1 (TCE and methane); ▲, M#2 (TCE and methane); •, M#3 (TCE and methane); ■, TCE Control (TCE without methane).

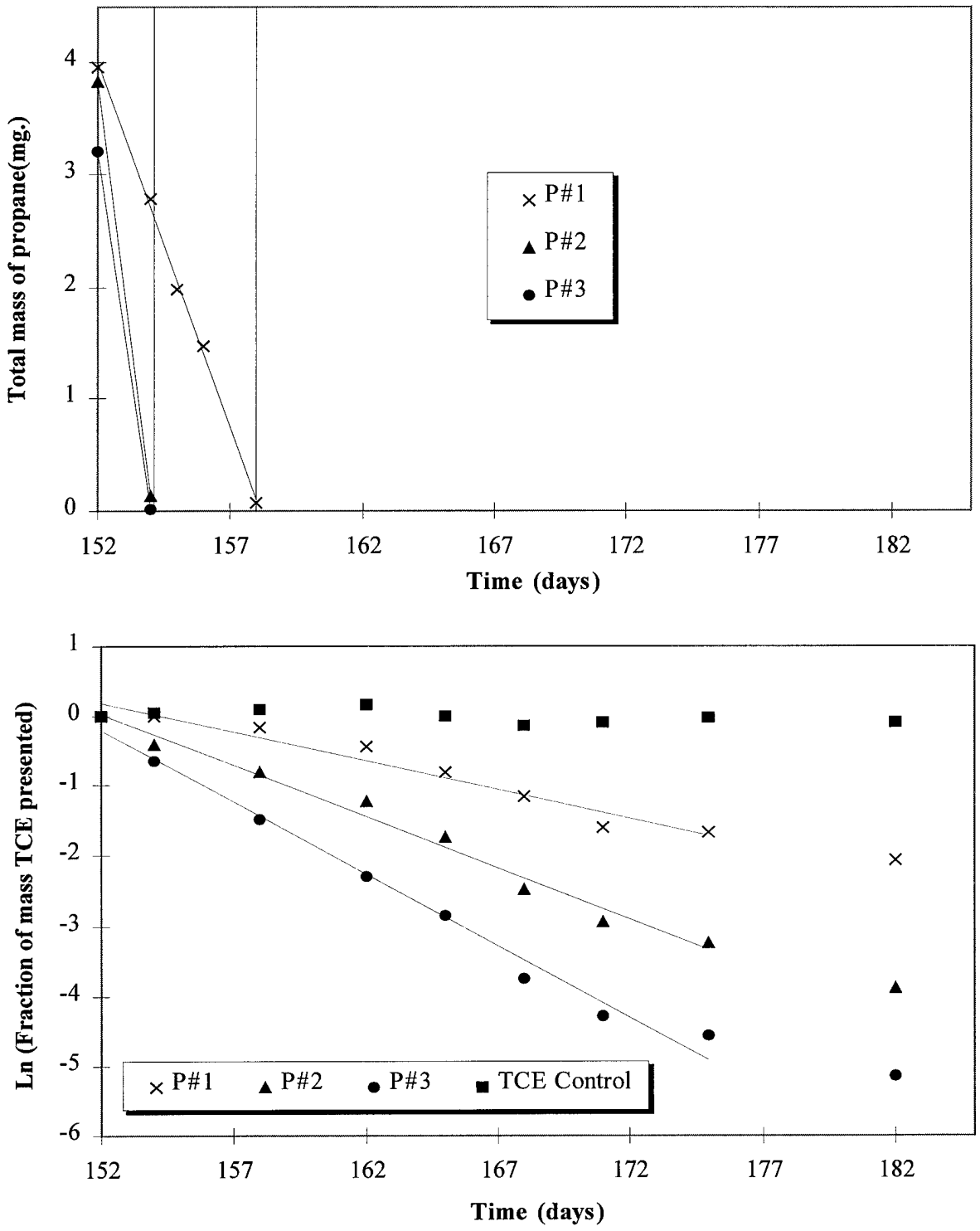


Figure 28. First order rate of TCE transformation and mass histories of propane consumption versus time. Symbols: x, P#1 (TCE and propane); ▲, M#2 (TCE and propane); •, M#3 (TCE and propane); ■, TCE Control (TCE without propane).

Table 12. Prolonged transformation of TCE after the primary substrate was consumed.

Growth substrate	Mixed enrichment cultures	a	Average Initial rates of TCE when primary substrate was being consumed (day^{-1})	Average first-order rates of TCE transformation after primary substrate was consumed (day^{-1})	Average period of TCE transformation after the primary substrates was consumed (days)
	M#1	10	0.143 ± 0.077	0.036 ± 0.01[b]	9.3 ± 4.0
Methane	M#2	9	0.211 ± 0.143	0.083 ± 0.06	10.8 ± 5.8
	M#3	11	0.180 ± 0.071	0.043 ± 0.02	9.7 ± 4.8
	P#1	6	0.096 ± 0.07	0.096 ± 0.07	15.5 ± 7.6
Propane	P#2	6	0.102 ± 0.06	0.102 ± 0.06	16.0 ± 9.8
	P#3	10	0.101 ± 0.07	0.101 ± 0.07	23.5 ± 4.7

a = number of estimates.
b = methane value is for after methane was consumed.

4. Effect of TCE concentration on the zero order rate of TCE transformation and methane or propane utilization

Methane and propane utilization rates and the initial TCE transformation rates were compared over the range of TCE concentrations studied. The zero-order rates are presented for comparison purposes. Figure 29 shows the effects of aqueous TCE concentrations on rates of methane and TCE transformations. As TCE concentrations were increased up to 4000 μg TCE/L in solution, the zero-order rates of TCE transformation increased as expected based on simple Monod kinetics. Methane-utilization rates were unaffected with TCE concentrations up to 2000 μg TCE/L. TCE transformation and methane utilization rates decreased in all the microcosms when aqueous TCE concentration were increased from approximately 5000 to 6000 μg TCE/L. The TCE concentration, TCE product toxicity, and competitive inhibition could potentially cause decreased rates. The original sterilized control, M#3, appears to be somewhat more tolerant to increasing TCE concentrations.

Figure 30 shows the effect of aqueous TCE concentration on the initial rate of propane utilization and TCE transformation. Upon exposure to elevated TCE concentrations, TCE transformation abilities were loss in propane microcosms (P#1 and 2). Propane microcosms (P#1 and 2) showed less TCE transformation and lower rates of propane utilization with increasing TCE concentrations. TCE transformation and propane utilization rates significantly decreased when aqueous TCE concentrations exceeded 2000 μg TCE/L. Microcosm (P#3), however, remained active toward TCE transformation despite the lower uptake rates of propane at the high TCE concentrations. TCE rates remained constant in P#3 after the TCE concentration reached 2000 μg TCE/L. Again, the most effective microcosm was the one that originally had been the sterilized control.

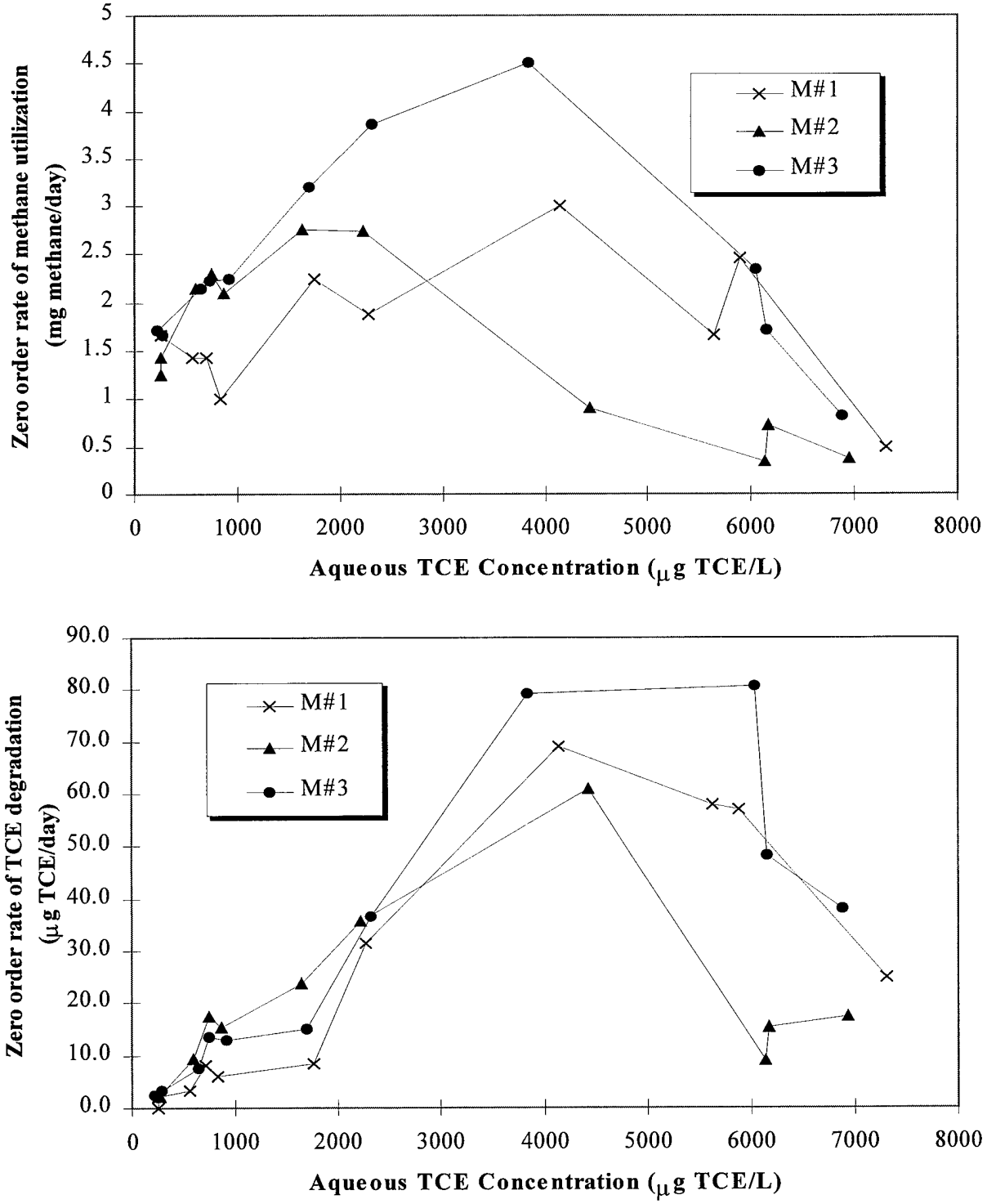


Figure 29. Initial rates of TCE transformation and methane degradation over a range of TCE concentrations during a one year period. Symbols: x, M#1 (TCE and methane); ▲, M#2 (TCE and methane); •, M#3 (TCE and methane).

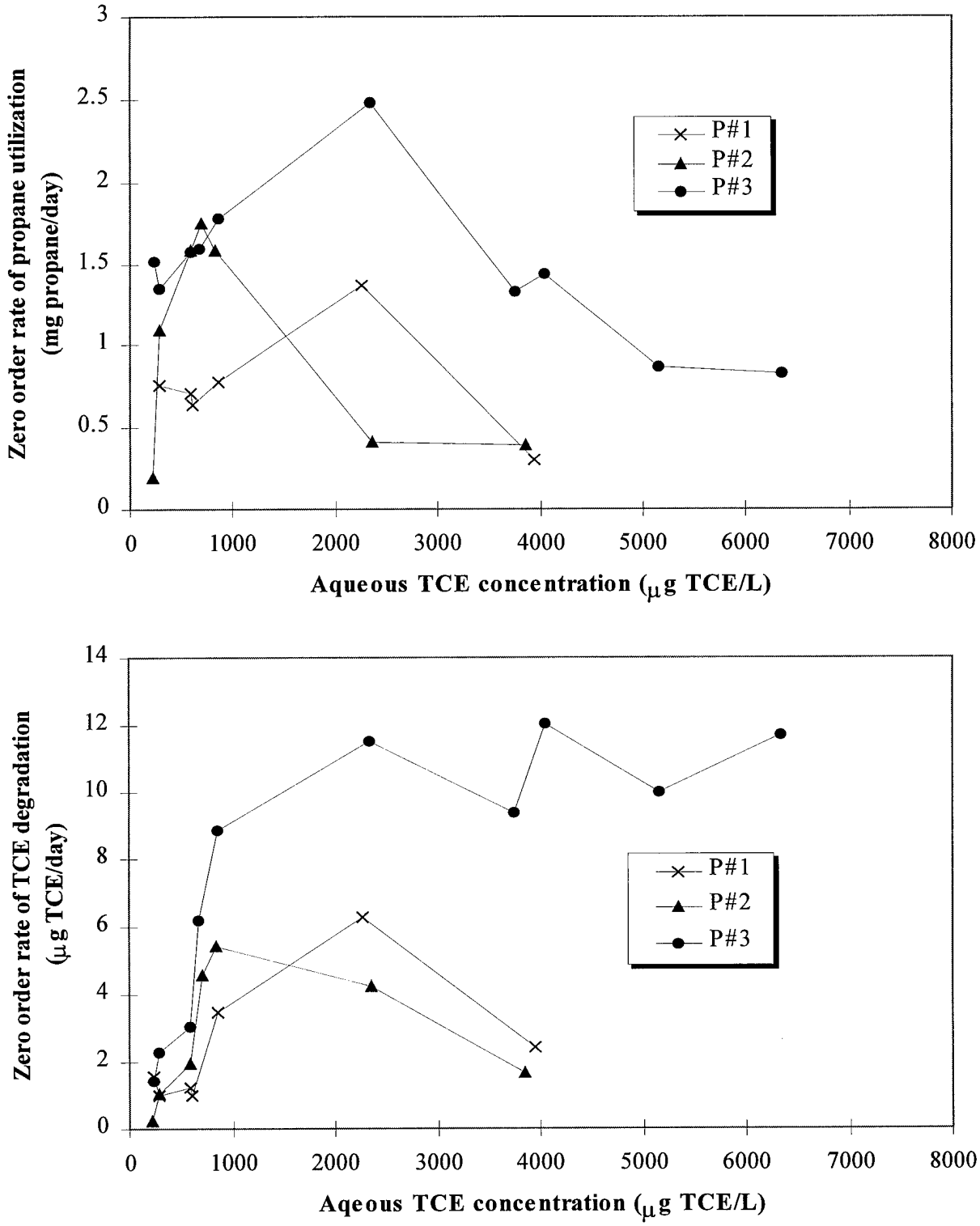


Figure 30. Initial rates of TCE transformation and propane degradation over a range of TCE concentrations during a one year period. Symbols: x, P#1 (TCE and propane); ▲, M#2 (TCE and propane); •, M#3 (TCE and propane).

Figure 31 shows the correlation of initial zero-order rates for methane utilization and TCE transformation at different TCE concentrations. The rate and extent of TCE transformation varied among methane microcosms and correlated well with the rate of methane consumption. A positive correlation between TCE transformation and methane utilization rates is shown with linear regression coefficients (R^2) of approximately 0.98. The slope also increases when aqueous TCE concentrations increase, consistent with Monod kinetics.

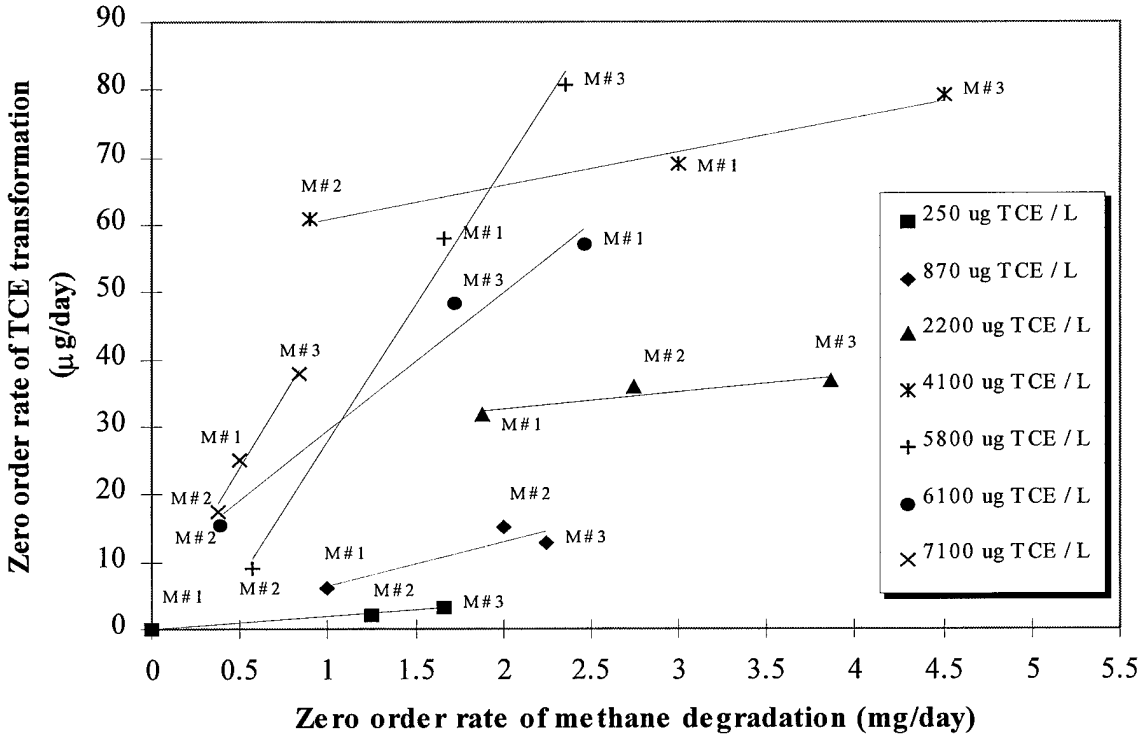


Figure 31. Effects of aqueous TCE concentration on the initial TCE transformation and methane utilization rates in three methane-utilizing microcosms. Solid lines are the linear regression best-fits at different aqueous TCE concentrations.

Figure 32 shows the correlation of initial zero-order rates of TCE transformation and propane utilization at different TCE concentrations. Results similar to those of methane were observed, but with lower linear regression coefficients (R^2) values of approximately 0.8. All the slopes were positive, indicating that the initial rate of TCE transformation again was dependent on the initial rate of propane utilization. The initial rate of TCE transformation by the propane-utilizers was much lower than for the methane-utilizers, even though the initial rates of propane and methane utilization were similar.

It is interesting to observe that even though propane has prolonged TCE activity, the initial rates of TCE transformation and propane utilization are correlated. The results also showed loss of TCE transformation ability of two propane-utilizers (P#1 and P#2) at high TCE concentrations, despite continued propane utilization. Only microcosm P#3 remained active with a high rate of TCE transformation and propane utilization. The

results presented in Figures 31 and 32 are not surprising, because the rates of cometabolism are expected to be correlated with the ability to utilize the primary substrate. These rates could be associated with higher microcosm populations, or higher enzyme activities, or different mixed cultures developing in the different microcosms.

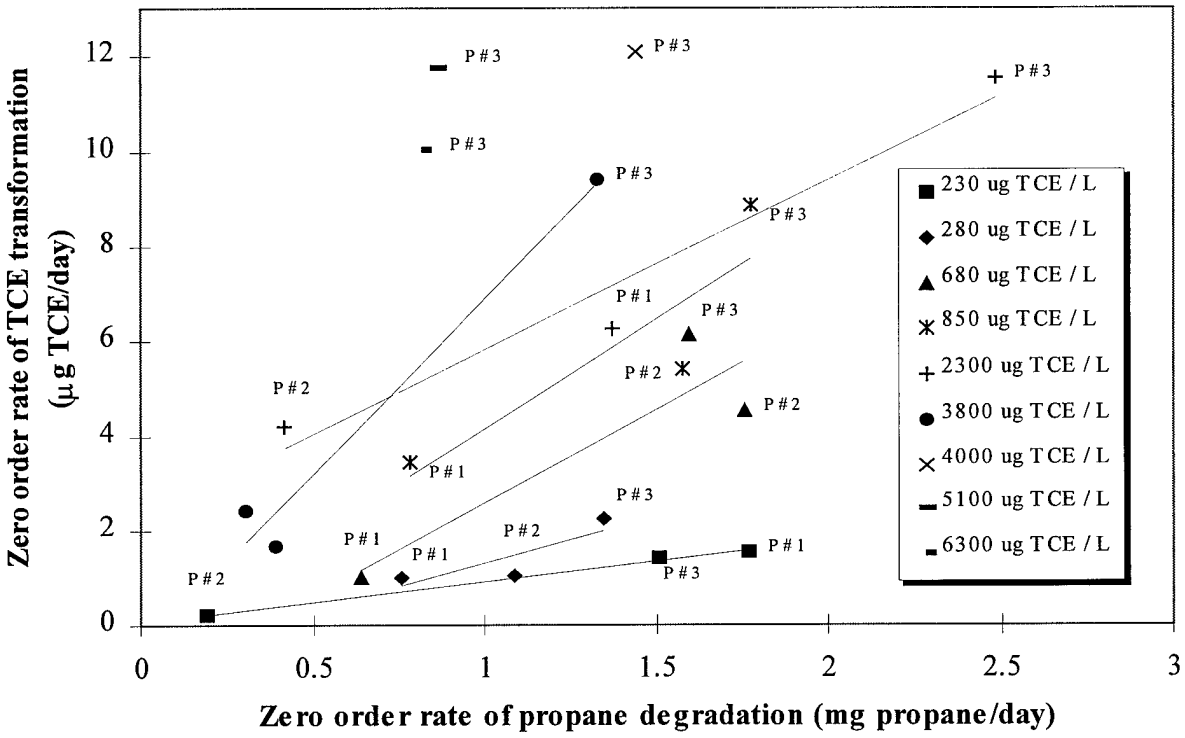


Figure 32. Effects of aqueous TCE concentration on the initial TCE transformation and propane utilization rates in three propane-utilizing microcosms. Solid lines are the linear regression best-fits at different aqueous TCE concentrations.

5. The ratio of initial TCE transformation rates to substrate consumption rates versus transformation yields by methane- and propane-utilizers

The correlations shown in Figures 33 and 34 indicate that the observed transformation yields are likely correlated with the ratios of initial TCE transformation rates to primary substrate utilization rates. The correlation between the ratio of TCE transformation rate to methane utilization rate versus transformation yields is shown in Figure 33. A linear relationship ($R^2 = 0.89$) was observed, when data that show competitive inhibition at high TCE concentrations were omitted. The linear relationship indicates the ratio of TCE rates to methane rates is directly proportional to transformation yields. Because the slope is approximately 0.50, the ultimate transformation yields for the methane-utilizers are about a factor of two greater than those based on the ratios of the rates. The difference may be attributed to slow TCE transformation activity after methane was consumed (Figure 27).

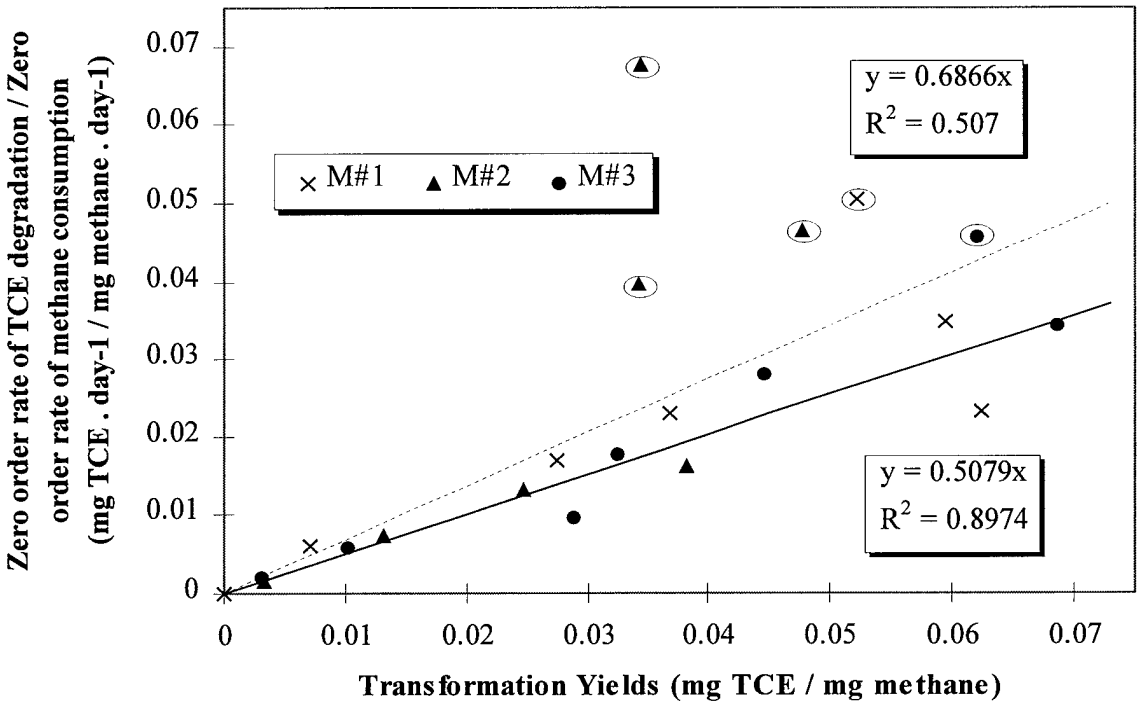


Figure 33. Ratio of initial TCE transformation rates to initial methane utilization rates versus transformation yields for methane microcosms where symbols : x, M#1, ▲, M#2, •, M#3. The circle results are whose at high TCE concentrations when competitive inhibition between methane and TCE was observed. The dashed line represents the linear regression fit using all the data. Solid line is the linear line with competitive inhibition data excluded.

Figure 34 shows the relationship between the ratio of initial zero-order TCE transformation rates to propane degradation rates versus transformation yields for the three propane microcosms. The linear correlation (R^2 was about 0.8) was achieved when data expressing competitive inhibition were omitted. An R^2 of 0.66 was observed when all data were included. The slope of the correlation ranged from 0.23 to 0.24. The transformation yields represented by the ratio of initial zero-order rates corresponded to only 20 to 30% of the observed transformation yields. This difference results from the long-term TCE activity after propane was consumed (Figure 28). It is interesting that the ratios of zero-order rate between TCE and propane degradation are correlated with the transformation yields of propane-utilizers, despite the fact that most of the transformation occurred after the propane was utilized. These results from the correlation of the rate of long-term activity to the initial rate of propane utilization previously discussed.

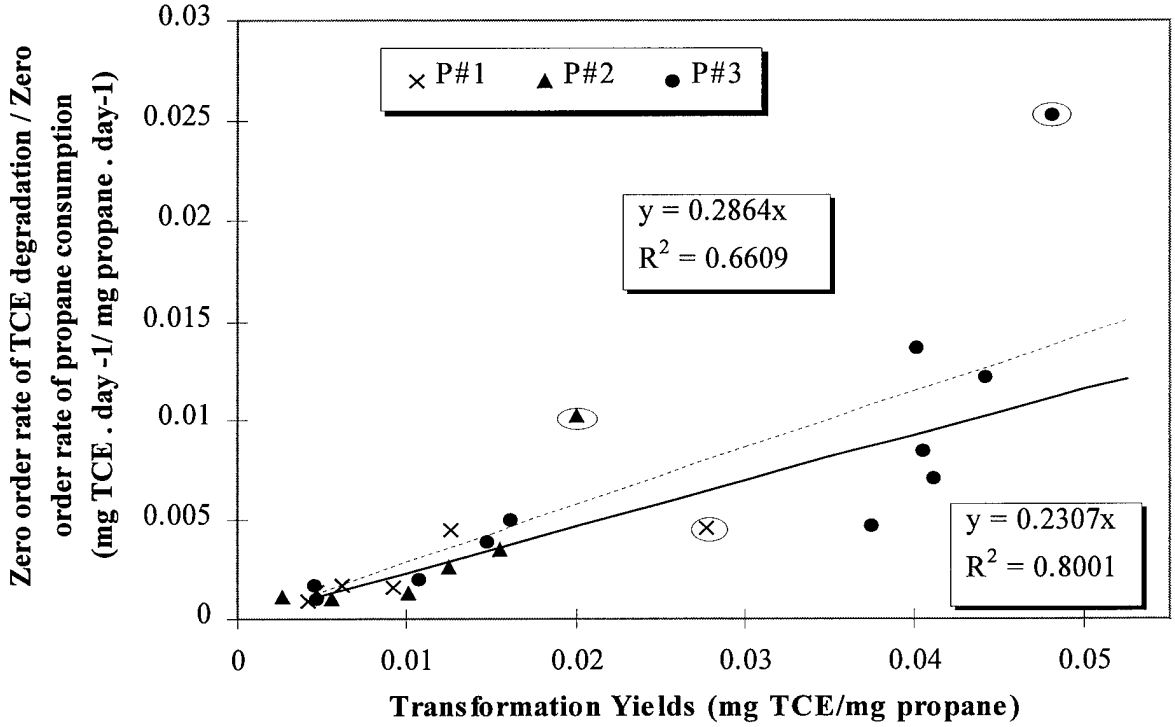


Figure 34. Ratio of initial TCE transformation rates to initial propane utilization rates versus transformation yields for propane microcosms {x P#1, ▲ P#2, • P#3}. The circle data are the results at high TCE concentrations when competitive inhibition between propane and TCE was observed. The dashed line represents the linear regression fit when all data is included. The solid line omits data that clearly showed competitive inhibition.

6. Transformation yields over a range of TCE concentrations by methane- and propane-utilizers

Figure 35 shows the transformation yields for three methane and three propane microcosms with increasing TCE concentrations over the 1-year period. Initially, two of the methane microcosms (M#1 and M#3) exhibited a lower TCE transformation yield than did microcosm (M#2), but after exposure to high TCE concentrations, the situation was reversed. The transformation yields of all methane cultures increased with time. The increase in transformation yields is likely due to increasing biomass with successive additions of growth substrate, and to the yields being limited by the TCE mass present at low TCE concentrations. The maximum transformation yield was 0.068 mg TCE/mg methane observed on methane microcosm M#3. Methane-utilizers were able to maintain high sustainable yields at high TCE concentrations.

Two propane microcosms (P#1 and P#2) showed a decreased ability to cometabolize TCE with increasing TCE concentrations above 2000 μg/L. The results suggest that long-term exposure to high TCE levels caused a loss in the cometabolic capabilities of the propane- utilizers. However, propane microcosms (P#3) remained active with increasing TCE concentration, yielding a maximum transformation yield of 0.048 mg TCE/mg propane.

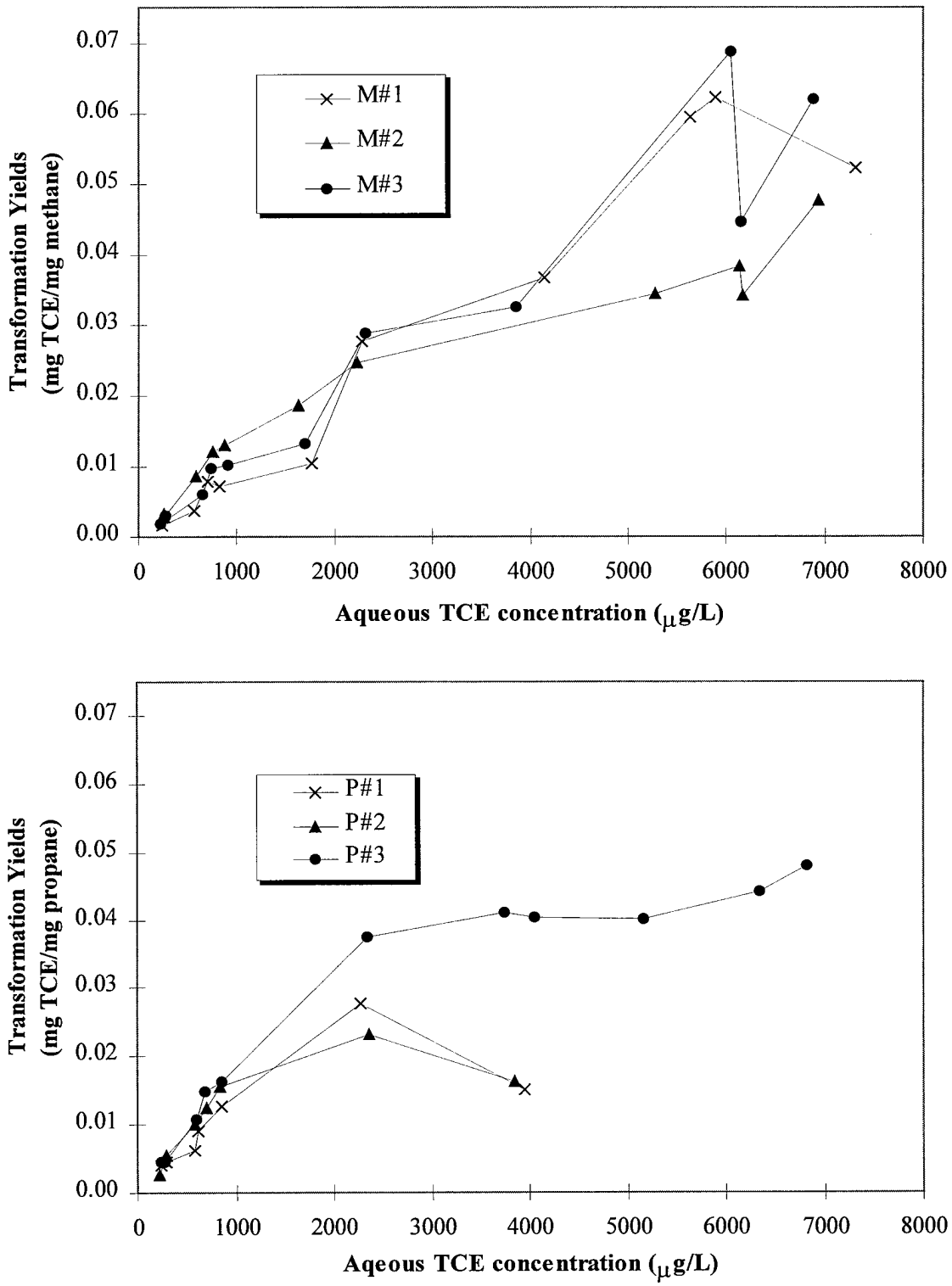


Figure 35. Ratio of mass of TCE transformed to mass of methane or propane consumed (T_y) as TCE concentrations were gradually increased over a 1-year period.

The gradual increase in TCE concentrations over a 1-year period resulted in different TCE transformation activities. The changes in TCE transformation ability in the methane and propane microcosms resulted from changes in TCE concentration, possibly caused by TCE product toxicity. Population shifts in the microcosm may have occurred, resulting in changes in TCE transformation ability. The results also indicate that methane-utilizers showed a better ability to cometabolize and cope with higher TCE concentrations than did the propane-utilizers.

7. Transformation of chloroform (CF) and 1,1,1-Trichloroethane (1,1,1-TCA) by methane and propane fed microcosm

The cometabolism of CF and 1,1,1-TCA by the methane- and propane-utilizers was tested. These compounds are found in the subsurface at McClellan AFB and, as with TCE, represent a chlorinated methane and ethane substituted with three chlorides. Transformation studies of individual CAHs were conducted in the presence of growth substrate in the microcosms previously challenged by TCE with 1,1,1-TCA incubation preceded by CF. Again, the mass of 1,1,1-TCA or CF was increased in the microcosms with a constant amount of growth substrate added. The maximum transformation yields for individual incubations with TCE, 1,1,1-TCA, and CF are shown in Figure 36. Propane-utilizers (P#1 and P#3) were more effective in transforming 1,1,1-TCA and CF than were the methane-utilizers.

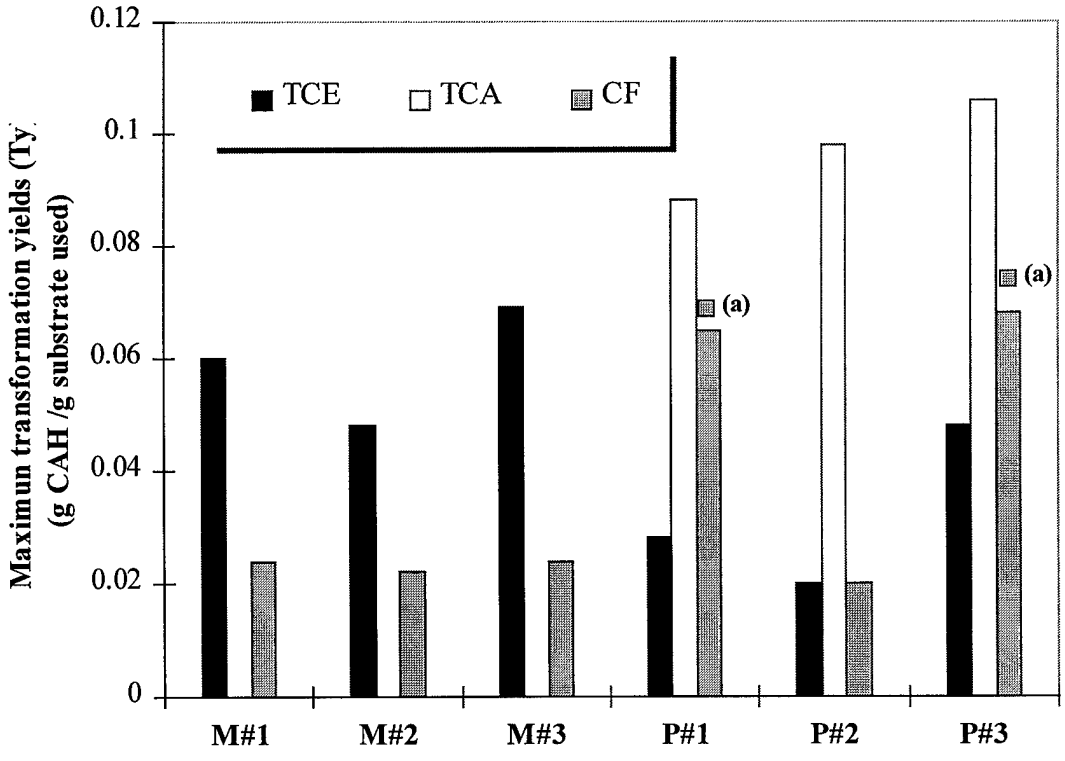


Figure 36. Maximum transformation yields (g CAH/g substrate) for TCE, 1,1,1-TCA, and CF achieved by methane- and propane-fed microcosms. (a): Transformation yields may have been limited by the mass of CAH present.

The methane-utilizers more effectively transformed TCE, but showed no ability to transform 1,1,1-TCA. Butane-utilizers showed no ability to transform any of the CAHs tested (data not shown). The transformation of CF appeared to inhibit both methane- and propane-utilizers. Furthermore, 1,1,1-TCA and CF transformation appeared to inhibit propane-utilizers more than methane-utilizers. Studies following chloroform transformation showed that propane-utilizers had a more difficult time recovering from their exposure to chloroform. Their difficulty may have resulted from propane-utilizers degrading more CF, so more product toxicity could have resulted.

8. Transformation of CAHs mixtures (TCE, 1,1,1-TCA, and CF) by methane and propane-utilizers

Studies of transformations of mixtures of TCE, 1,1,1-TCA, and CF were then performed at CAH concentrations of 1 mg /L for each compound. The results from the methane microcosm M#3 are presented in Figure 37. TCE was the most effectively transformed followed by CF. No transformation of TCA was observed. These observations are consistent with the results from the individual compound test. Methane utilization appeared to be inhibited by TCE and CF transformation, because slower methane uptake rates were observed in all the microcosms. However, transformation of TCE and CF were more rapid during methane degradation. In the absence of methane, the culture continued to degrade TCE and CF at slower rates for several days after methane was consumed.

The transformation of mixtures of CF, 1,1,1-TCA, and TCE in the propane microcosm P#3 is shown in Figure 38. Effective transformation of CF, TCA, and TCE were observed. CF was most rapidly transformed and followed by TCA and TCE. Complete transformation of 1.0 mg/L of CF (68 μg total mass of CF) and TCA (82 μg total mass of TCA), and nearly complete transformation of TCE (70 μg total mass of TCE) in aqueous solution were observed. Transformation of CF, TCA, and TCE was observed during propane utilization, but transformation rates were most rapid after the propane was reduced to lower concentrations, suggesting competitive inhibition of propane on CAH transformations. The long lag time may have been caused by competitive inhibition of CF on propane utilization.

Similar results were observed in microcosm P#1, but not for propane microcosm P#2. Propane-utilizers in microcosm P#2 showed no transformation of mixed CAHs tested, even though this culture previously transformed the individual CAH. It was unclear that why the transformation of CAH mixtures were not observed. A possible explanation is that the transformation on CF in the prior study diminished the cometabolic potential of the P#2 microcosm.

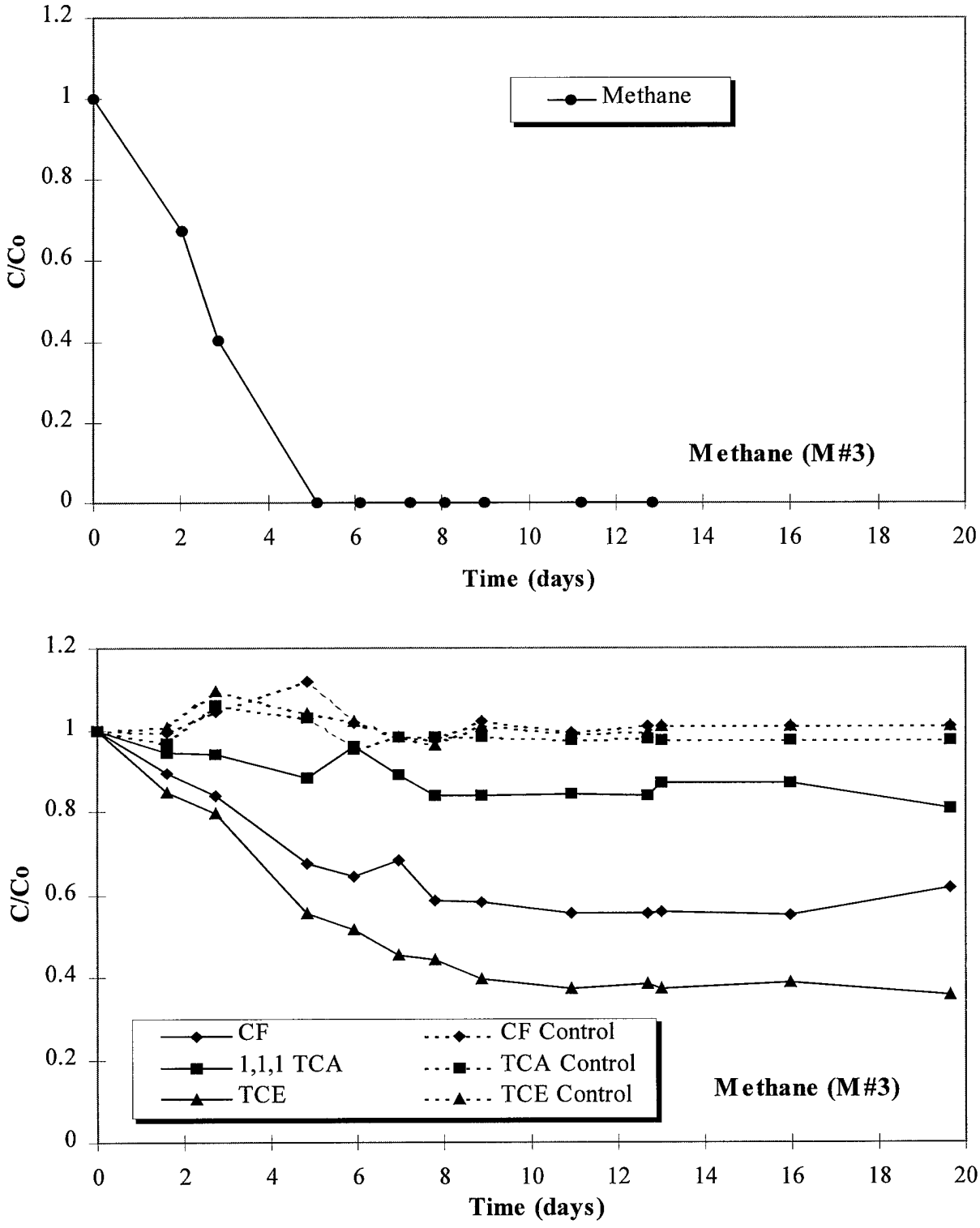


Figure 37. Methane utilization and CAH transformation in the methane microcosm M#3.

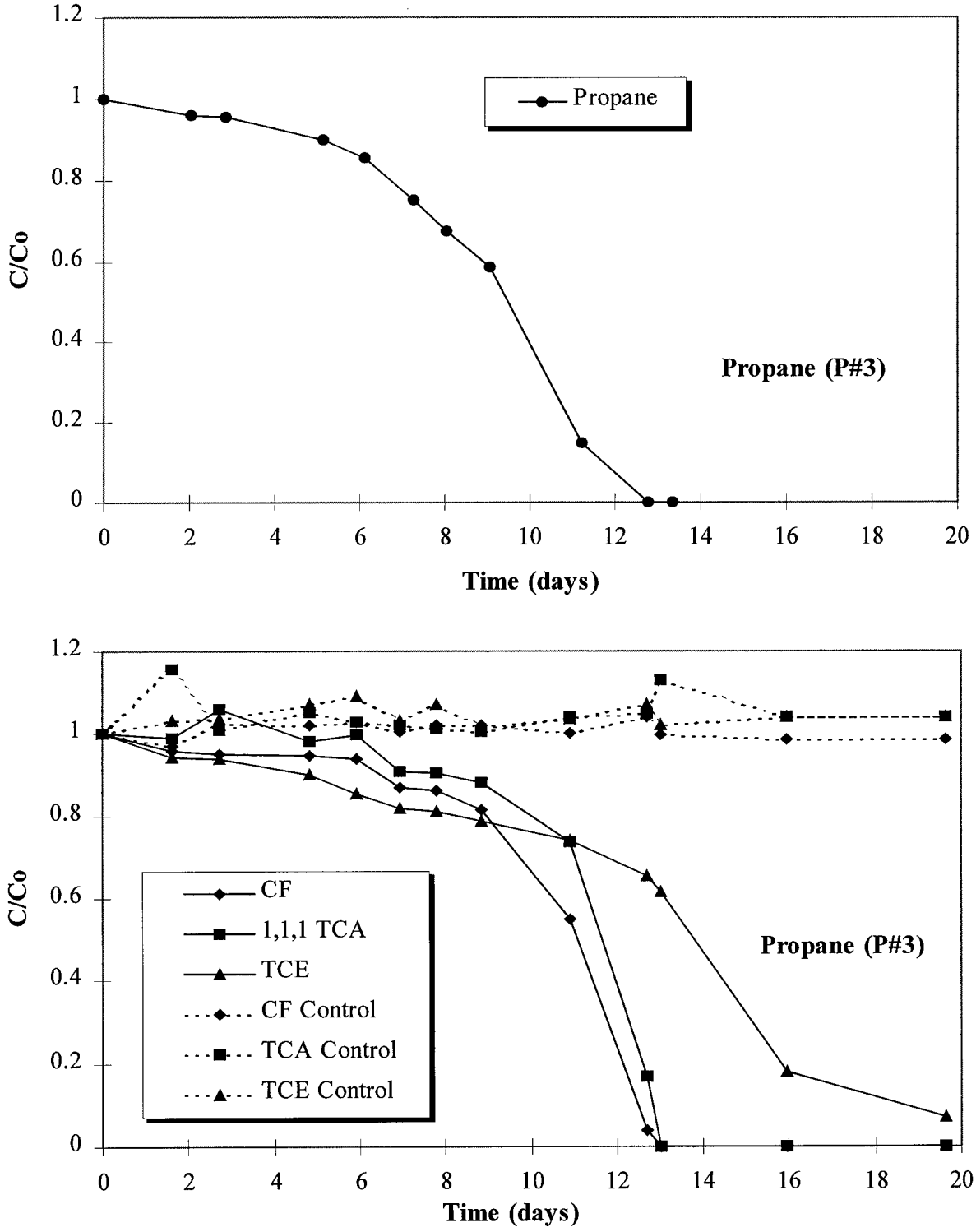


Figure 38. Propane degradation and CAH transformation in the propane microcosm P#3.

A comparison of transformation yields for the CAH mixture tests are shown in Figure 39. The methane-utilizers were able to transform CF and TCE, but not 1,1,1-TCA. Higher transformation yields for TCE compared to CF were observed and consistent with the results for single compounds. Propane-utilizers effectively transformed 1,1,1-TCA, CF, and TCE. The T_y results represented conservative estimates, since complete transformation of CAH mixtures was observed. No transformation of CAHs was observed in microcosm P#2. For two effective cultures, P#1 and P#3, the observed transformation yields of the propane-utilizers with mixed CAHs transformation were much higher than that of methane-utilizers. It is interesting that TCE was more effectively transformed in the active microcosm compare to those of methane microcosm, compared to test with TCE alone. Here the simultaneous transformation of CF and TCE appears to have a greater impact on methane-utilizers than propane-utilizers.

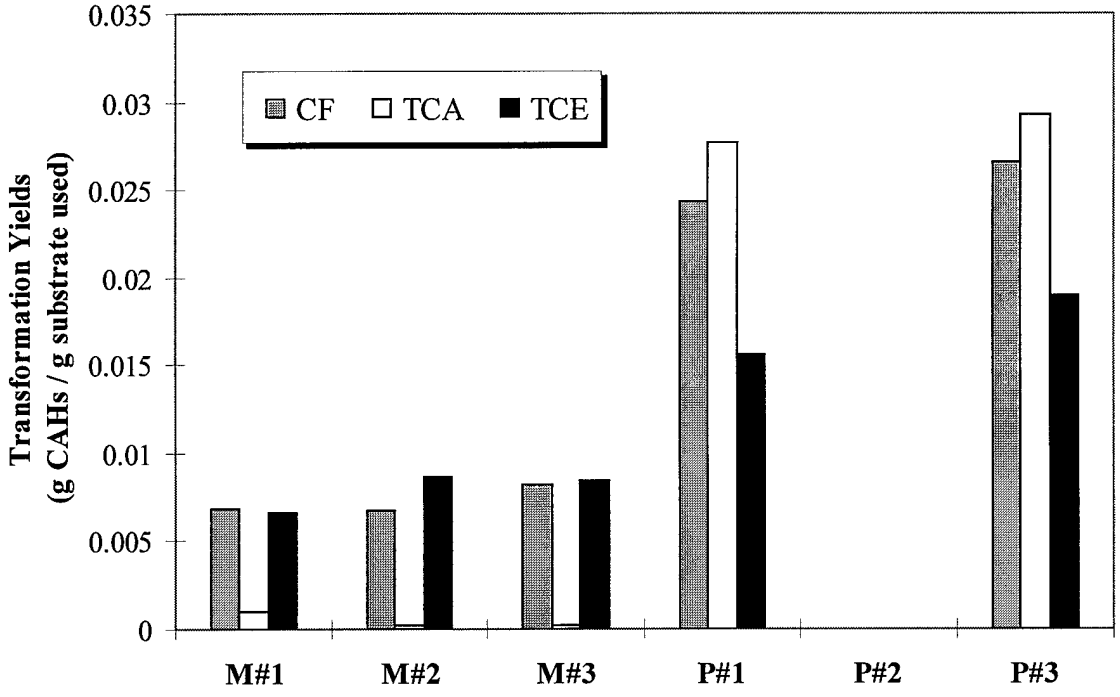


Figure 39. Comparison of transformation yields for mixed CAHs by methane and propane-utilizers.

D. DISCUSSION

The ability to stimulate microbes on methane, propane, and butane was demonstrated in the microcosms constructed with McClellan AFB subsurface solids and groundwater. The study showed that McClellan subsurface appears to have a diverse microbial community. Stimulation of propane- and butane-utilizers had about twice the lag period of the methane-utilizers. The longer lag times indicate either lower numbers of these microorganisms in the McClellan AFB subsurface or slower growth rates. The results indicated that propane and butane are readily stimulated in the McClellan AFB subsurface and might be useful for bioremediation of CAHs.

Methane- and propane-utilizers were able to transform TCE, whereas butane-utilizers had no ability to stimulate TCE cometabolism. It is not known why butane-utilizing microorganisms were unable to degrade TCE. Previous studies indicated that butane-utilizing microorganisms, *Pseudomonas butanovora*, isolated from an oil-refining plant, were unable to utilize normal alkene compounds (McLee et al., 1972). However, in another study, butane-utilizing bacteria were able to cometabolize TCE and CF (Kim et al., 1997).

After a long-term incubation with TCE, three methane and three propane-fed microcosms exhibited different abilities to transform TCE. The observed differences in TCE transformation may be due to different inherent abilities among the cultures to degrade TCE. However, the rate of substrate consumption among cultures correlated well with the rate and extent of TCE transformation. Higher rates of substrate consumption corresponded to higher rates of TCE transformation. Similar results were observed in a study of eight mixed methane-utilizers (Broholm et al., 1993). They revealed that the ability of mixed cultures of methane-utilizing bacteria to degrade TCE varied significantly, even though the cultures were grown under the same conditions.

Our observations show prolonged TCE transformation activity by propane-utilizing microorganisms after the propane was consumed. TCE transformation followed first-order kinetics for about 3 to 4 weeks after propane was consumed. In contrast, methane-utilizers showed a high TCE transformation rate when methane was consumed, and then transform TCE at a slower rate for about 1 week after methane was consumed. The reason for prolonged TCE activity of propane-utilizers is unknown. It may be that by-products of propane oxidation possibly serve as an alternative energy source to further transform TCE after metabolic degradation of propane is utilized. The propane-utilizers might also effectively store energy reserves that are used later to drive the TCE transformation.

Previous studies showed that formate and methanol, catabolic intermediates of methane oxidation, could be used as alternative energy sources to increase and extend TCE transformation in pure and mixed methane-utilizing cultures (Alvarez- Cohen and McCarty, 1991b; Brusseau et al., 1990; Henry and Grbic-Galic, 1991; Oldenhuis et al., 1991; Semprini et al., 1991). Energy reserves stored during growth-limited conditions of methane-utilizers are another possible reason for the prolonged TCE activity. Previous observations have suggested that the energy reserves (as poly-β-hydroxybutyrate [PHB]) of methane-utilizers (Dawes and Senior, 1973) can provide intracellular reducing

equivalents to extend and improve TCE transformation (Henrysson and McCarty, 1993; Henry and Grbic-Galic, 1991).

A similar explanation can be postulated for the long-term TCE activity of the propane-utilizers. Intermediate byproducts associated with catabolism of propane may provide an alternative energy source to drive TCE transformation. Previous studies have shown that propane-grown microorganisms are able to degrade a broad range of aliphatic hydrocarbons including short-chain alkenes (Hou et al.,1983; McLee et al., 1972). Intermediate byproducts from metabolic degradation of propane have been proposed (Perry, 1979; Stephen and Dalton, 1986). Acetone is formed, which is further oxidized from 2-propanol (Perry, 1980). Acetone was shown to be an excellent carbon and energy source for propane microorganisms. The organisms isolated by enrichments of acetone as substrate normally were able to oxidize propane (Lukin and Foster, 1963). However, it may be that propane-utilizing microorganisms can effectively store more energy reserves than methane-utilizers. The synthesis of a copolymer of PHB by propane-utilizing microorganisms has been reported (Davis, 1964). These propane species also can synthesize aliphatic waxes during growth on propane as substrate. More research is needed to determine what is causing the prolonged TCE activity of propane-utilizers. It is also interesting that long-term TCE activity was achieved with aquifer solids and the background groundwater chemistry. To our knowledge, this is the first observation of such prolonged activity under groundwater conditions.

Changes in the ability to transform TCE were observed with both methane and propane-utilizers as the TCE concentration was increased over a 1-year period. High TCE concentrations caused inhibition of methane and propane utilization. Lower rates of substrate utilization and TCE transformation were observed when TCE concentrations reached 5 mg TCE/L and 2 mg TCE/L, respectively, in methane and propane microcosms. The propane cultures in microcosm P#3 exhibited decreased TCE transformation rates above 2 mg TCE/L, even though lower propane utilization rates were observed. Except for microcosm P#3, all methane-utilizers showed a better ability to transform and cope with higher TCE concentrations than the propane utilizers. The sterilized control microcosms (M#3 and P#3) may have been inoculated by the filtered groundwater being replaced in the control microcosms. Small size microorganisms passing through the 0.45-μm filter may have been stimulated. If so, microbes that better tolerate TCE product toxicity may have been selected. More experiments are required to confirm this possibility.

The ratios of zero-order TCE transformation rates to substrate utilization rates were correlated with transformation yields for TCE in methane-utilizers and propane-utilizers. The ratios yielded about 50% of the observed transformation yields for methane-utilizers and 20 to 30% of the transformation yields for propane-utilizers. The long-term TCE activity with propane-utilizers resulted in the lower percent. Even though the zero-order TCE transformation rates for the methane-utilizers were much higher than for the propane-utilizers, the maximum transformation yields on methane- and propane-utilizers were similar, resulting from the long-term activity of the propane-utilizers.

Studies of resting cells and of mixed and pure cultures have provided different transformation yields of TCE for specific cometabolic substrates (Chang and Alvarez-Cohen, 1995; Dolan and McCarty, 1993; Wilcox et al., 1995). Table 13 compares the methane and propane results from previous studies. The transformation yields for TCE observed in our study with both methane- and propane-utilizers are in the range of those observed in prior studies. However, resting cell transformation yields and transformation yields in the presence of growth substrate, cannot be compared directly.

Table 13. Comparison of transformation yields for TCE (mg TCE/ mg primary substrate).

CONDITION	SOURCE	TRANSFORMATION YIELDS
Methane-utilizers (resting cells)	Dolan and McCarty, 1993	0.040
Methane-utilizers (resting cells)	Change and Alvarez-Cohen, 1995	0.017
Methane-utilizers (resting cells)	Change and Alvarez-Cohen, 1996	0.180
Methane-utilizers	reported here	0.068
Propane-utilizers (resting cell)	Change and Alvarez-Cohen, 1995	0.005
Propane-utilizers	Wilcox et al., 1995	0.005
Propane-utilizers	reported here	0.048

The microcosms exhibited TCE transformation activity with gradual increases in TCE concentrations over a 1-year period. Two of the propane microcosms (P#1 and 2) eventually recovered TCE transformation abilities after reducing the TCE concentration. Indigenous microbes therefore remained active toward TCE transformation and substrate utilization for prolonged periods of stimulation.

The effectiveness of TCE cometabolism by indigenous phenol-fed microorganisms declined significantly during a 280-day experiment (Munakata et al., 1997). However, the results from our study and the results from the phenol column microcosm study cannot be compared directly. Much more TCE was transformed in phenol column microcosm studies that may have generated higher TCE transformation product toxicity, causing more significant inactivation. Jenal-Wanner and McCarty (1997) have recently reported no loss of TCE transformation ability in the microcosms stimulated on phenol or toluene and challenged with TCE over a 1-year period.

The results presented in this study have demonstrated that indigenous microorganisms grown on propane are capable of transforming TCE, CF and 1,1,1 TCA. Microorganisms grown on methane transformed TCE and CF, but not TCA. Butane-utilizers did not transform any of the CAHs tested. Methane-utilizers exhibited the highest transformation yields for TCE. The propane-utilizers more effectively transformed CAH mixtures.

TCA transformation studies indicated that propane utilization was strongly inhibited by TCA transformation. CF transformation product toxicity likely occurred for both methane and propane-utilizers. The decrease of methane and propane utilization rates occurred after exposure to CF. CF transformation product toxicity or high competitive inhibition between CF and growth substrate are possible reasons for the decreased rate. Previous research has shown that CF transformation product toxicity to methane-utilizers, decreasing their ability to transform CF (Broholm et al., 1990; Alvarez-Cohen and McCarty, 1991b; Oldenhuis et al., 1989 and 1991). However, propane-utilizer exhibited much higher CF transformation yields than methane-utilizers, indicating that the propane-utilizers stimulated here have higher ability to cometabolize CF.

The propane-utilizers were much more effective at transforming 1,1,1-TCA and CF. Propane utilizations were inhibited by 1,1,1-TCA. CF transformation product toxicity likely occurred for both methane- and propane-utilizers. The decrease of methane and propane utilization rates occurred after exposure to CF. Two possible reasons for the decreased rate are the CF transformation product toxicity or the high competitive inhibition between CF and the growth substrate. Previous research had demonstrated CF transformation product toxicity to methane-utilizers, decreasing their ability to transform CF (Broholm et al., 1990; Alvarez-Cohen and McCarty, 1991b; Oldenhuis et al., 1989 and 1991). However, the propane-utilizers exhibited much higher CF transformation yields than did the methane-utilizers. The results from this work indicate the methane-utilizers have better potential for TCE transformation, but propane-utilizers appear to be better suited to transforming mixtures of CAHs. The long-term activity of the propane utilizers might be used to optimize in situ remediation because substrates do not need to be continuously fed for transformation to occur.

The transformation of CAH mixtures (TCE, CF and 1,1,1-TCA) resulted in higher transformation yields for CAHs by propane-utilizers than the methane-utilizers. Propane-utilizers were much more effective to transforming CAH mixtures. Thus, indigenous propane-utilizers from the McClellan subsurface appear to have a better potential for in situ bioremediation of groundwater contaminated with CAH mixtures. Because CF and 1,1,1TCA are the contaminants that have been detected along with TCE in the McClellan AFB, stimulation of propane-utilizers would be desirable for in-situ bioremediation at this site.

The results indicated that the presence of CF and 1,1,1 TCA in the groundwater are of greater concern when methane-utilizers are stimulated for TCE transformation. CF lowers the TCE transformation ability of methane-utilizers. This would be a major concern when cometabolic TCE degradation by methane cultures are used in clean up processes. Lower observed transformation yields for TCE are likely when CF is present. However, the propane-utilizers had higher ability to transform TCE than methane-utilizers in the presence of CF and TCA. Higher TCE transformation ability of propane-utilizers in the presence of mixed CAHs possibly results from faster removal of CF than TCE, with TCE transformation occurring after CF is removed. The processes causing this behavior need to be investigated in much more detail.

SECTION V

THE EFFECT OF NITRATE ON TCE COMETABOLISM BY METHANE AND PROPANE UTILIZING MICROORGANISMS STIMULATED FROM MCCLELLAN AFB SUBSURFACE

A. INTRODUCTION

Nutrient requirements are one of the major factors that influence the potential for chlorinated aliphatic hydrocarbon (CAH) transformation in situ. Nutrients are needed for maintaining the growth of subsurface microorganisms that cometabolize TCE and other CAHs. Nitrogen is one of the most essential nutrients that can be limiting in groundwater, with nitrate usually being the available nitrogen source. The addition of a nitrogen source such as nitrate or ammonia to the nitrogen-deficient subsurface may be required to enhance TCE and CAH cometabolism.

Methane-utilizing bacteria have been extensively studied for 25 years. Methane-utilizing microorganisms are widespread in transition zone between aerobic and anaerobic zone in the subsurface where methane and oxygen are present (Hanson, 1980). Methane-utilizers are categorized into two groups (Type I and II) based on their internal membranes. Both types can express a particulate enzyme form called particulate methane monooxygenase, (pMMO). Only Type II methanotrophs can express soluble methane monooxygenase, (sMMO) that can cometabolize a broad range of substrates, including TCE and many other CAHs. Previous studies have shown that soluble forms of MMO can be produced under the copper limited growth conditions, while Type I organisms that express pMMO require copper for growth (Brusseau et al., 1990; Oldenhuis et al., 1989; Tsien et al., 1989). Recent studies have also found that methanotroph Type II that efficiently cometabolize TCE can also fix nitrogen under conditions of low oxygen tension (De Bont, 1976; Murrell and Dalton, 1983; Chu and Alvarez-Cohen, 1996; Graham et al., 1993).

Type II methanotrophs appear to be selected under nitrogen-limiting conditions, while Type I strains appear to be present under all methane-enrichment conditions when nitrate or ammonia are available (Dugan et al., 1978; Graham et al., 1993). Graham et al., (1993) found that *M. Trichosporium* OB3b, Type II strains, can be selected under conditions of limiting nitrogen and low oxygen tension. Type II methanotrophs typically fix nitrogen, while Type I organisms are unable to fix molecular nitrogen and nitrate, ammonia or organic nitrogen for growth.

Nitrogen-fixing methane-utilizers expressing sMMO, grown at low oxygen tensions, were able to degrade TCE rapidly with a high transformation capacity (Chu and Alvarez-Cohen, 1996). These results indicate that reactors can be used to manipulate methane-utilizing bacteria species selection to optimize TCE and other CAH removal. Nitrate is one of the primary factors influencing methanotrophic species selection. On the other hand,

most research claims that expression of sMMO under in situ conditions may be difficult due to copper availability in the subsurface. Thus, it might be difficult to control conditions to select of Type II dominant species on the subsurface environment (Graham et al., 1993).

In the absence of growth substrate or external electron source, methane-utilizers can also produce poly-β-hydroxybutyrate (PHB) as an endogenous electron donor or source of required reducing power. PHB is an intracellular reserve polyester polymer whose synthesis serves as an electron sink in microorganisms grown under limited conditions (such as limitations of N, P, S, Mg, and/or O_2) (Dawes and Senior, 1973). PHB may be used for the regeneration of NADH during TCE transformation (Asenjo, J. A. et al., 1986; Henrysson and McCarty, 1993). Intracellular reducing equivalents to improve and extend TCE transformation might be due to the catabolism of stored PHB contents in methane-utilizers. A positive correlation was observed between PHB contents and the naphthalene oxidation rate (a measure of soluble MMO activity), as well as between PHB and the TCE transformation rates and capacity (Henrysson and McCarty, 1993; Henry and Grbic-Galic, 1991). High accumulation of PHB was also observed upon depletion of nitrate in *Methylosinus trichosporium* OB3b cultures, Type II strains (Shah et al., 1996).

The goal of this study was to determine the effect of nitrate on methane and propane-utilizers stimulated on McClellan AFB aquifer solids. The effect of nitrate on TCE cometabolism was determined in batch microcosms with groundwater and aquifer solids. A comparison of the effect of nitrate on methane and propane enrichment cultures was investigated in the batch microcosms with groundwater or media, without aquifer solids present.

B. MATERIALS AND METHODS

1. Indigenous microcosm studies with aquifer solids

The studies were performed in batch microcosms constructed with aquifer material and groundwater from McClellan Air Force Base. Methane, propane and butane were used as growth substrates for each of microcosm studies. The microcosm method was adapted from Broholm et al., (1990) and Yi Mu and Scow, (1994). The microcosms were constructed using 125-mL amber serum bottles (Wheaton Class Co., Millville, NJ.). Aquifer material from the McClellan Air Force Base, Sacramento, CA, was wet sieved with site groundwater under a laminar flowhood using a No. 8 sieve (2.38 mm opening) to remove large particles. The site groundwater was filtered (0.45-μm sterilized filter) before use. 15-mL of wet solids and 50-mL of filtered ground water were added to each batch microcosm, leaving a 60-mL air-filled headspace as a source of oxygen. The headspace permitted sampling of the gaseous substrate, oxygen, and TCE. The microcosms were crimp sealed with a Teflon™ butyl rubber cap (Kimble Co., IL), then inverted and incubated at room temperature on a shaker table at 100 rpm.

Batch microcosms were prepared and operated as described previously. Active methane (M#1, 2, and 3), propane (P#1, 2, and 3), and butane microcosms (B#1, 2, and 3) were used to study the effect of nitrate addition on substrate utilization and TCE cometabolism. The exchange of 50/50 groundwater was performed in the methane (M#2 and 3), propane (P#1 and 3), and butane (B#2 and 3) microcosms prior to the addition of the growth substrate and TCE. The groundwater was amended with nitrate (30 mg/L), since nitrogen (as nitrate) was found to be limiting in the groundwater. Groundwater exchanges lacking nitrate were performed in microcosms, M#1, P#2, and B#1. Nitrate-limited microcosms were used to compare TCE transformation and substrate utilization with nitrate-rich microcosms. Groundwater exchange procedure was performed as described in the previous study.

2. Enrichment microcosms with media and groundwater

Mixed methane and propane enrichment cultures used in this study were obtained from batch microcosm studies on McClellan AFB. Methane and propane enrichment cultures were obtained by transferring 1 ml of groundwater and aquifer solids from the previous stimulate microcosm M#1 and P#3. The enrichment cultures were grown in the media, containing; 15 mM K_2HPO_4 + NaH_2PO_4 (a buffer 7.5 solution), 0.5 mM $MgSO_4$, 0.1 mM $CaCl_2$, 23.5 mM $NaNO_3$, 0.796 mM $(NH_4)_2SO_4$, 0.1 g of yeast extract, and trace element solution containing ; 22.6 μM $FeSO_4.7H_2O$, 1.52 μM $MnCl_2.4H_2O$, 0.51 μM $ZnSO_4.7H_2O$, 1.0 μM H_3BO_3, 0.45 μM $Na_2MoO_4.7H_2O$, 0.1 μM $NiCl_2.6\,H_2O$, 0.1 μM $CuCl_2.2H_2O$, and 0.1 μM $CoCl_2.6H_2O$. Each of suspended cultures in 750-mL serum bottles were then shaken and incubated at 30°C on a shaker table at 190 rpm. Each of 750-mL serum bottles was supplied by methane or propane (10 % in the headspace) to sustain the microbial growth before use in the experiments.

Enrichment cultures were used to study the effect of nutrient addition on TCE cometabolism. Three different media compositions were tested included; 100 % media, 100 % groundwater, and 50/50 % media /groundwater. Three serum bottles were constructed for each substrate tested. The 125-mL serum bottles contained 60 ml of medium were inoculated with 1 ml of the enrichment cultures, growth substrate and TCE. The serum bottles were then crimp sealed with a Teflon™ butyl rubber (Kimble Co., IL) then inverted and incubated at room temperature on a shaker table at 100 rpm. The serum bottles were maintained with repeated additions of growth substrate and TCE for a period of 150 days without exchanging groundwater or adding nutrients.

3. Analytical methods

Methane, propane, butane, TCE, and oxygen were determined by headspace sampling of the microcosms as previously described in Section IV. The nitrate concentration was determined using 50-μL aqueous sample as previously described in Section IV.

C. RESULTS

1. The effect of nitrate addition on indigenous microcosm studies with aquifer solids

The batch microcosm studies with aquifer solids were conducted to determine the effect of nitrate addition on indigenous microbial activities and TCE cometabolism. Methane, propane and butane were used as growth substrates for each of the microcosm studies. Active methane (M#1, 2, and 3), propane (P#1, 2, and 3), and butane microcosms (B#1, 2, and 3) were used to study the effect of nitrate addition on substrate utilizations and TCE cometabolism.

The uptake of methane and propane, TCE transformation, and the effect of nitrate addition are shown in Figures 40 and 41. In active microcosms, complete methane and propane utilization required about 2 to 5 days of incubation. TCE removal was observed in nitrate amended microcosms, M#2, M#3, P#1, and P#3. Methane and propane microcosms M#1 and P#2 showed limited microbial activity under nitrate limited conditions during the first incubation with substrate and TCE. No uptake of methane and TCE transformation was observed in microcosm M#1, while microcosm P#2 showed slow utilization of propane and TCE transformation. Nitrate was amended into M#1 and P#2 upon second additions of methane, propane, and TCE. More rapid removal of methane, propane and TCE was observed, with similar methane and propane removal rates achieved in all the microcosms. TCE transformation was greatly enhanced in microcosm M#1 and P#2 that were initially nitrate limited. The results indicated that nitrate was a limiting nutrient in the site groundwater and the nitrate addition is necessary for effective TCE transformation. The results also indicated that enhancing rates of methane and propane uptake were associated with enhanced rates of TCE transformation.

Figure 42 shows the effects of nutrient addition on butane utilization and TCE cometabolism. None of the butane microcosms transformed TCE, with or without nitrate addition. Butane utilization, however, was also enhanced with nitrate addition. Nitrate addition therefore increased butane utilization, but not TCE transformation. No TCE transformation occurred even after several additions of butane, confirming TCE was not being transformed by butane-utilizers.

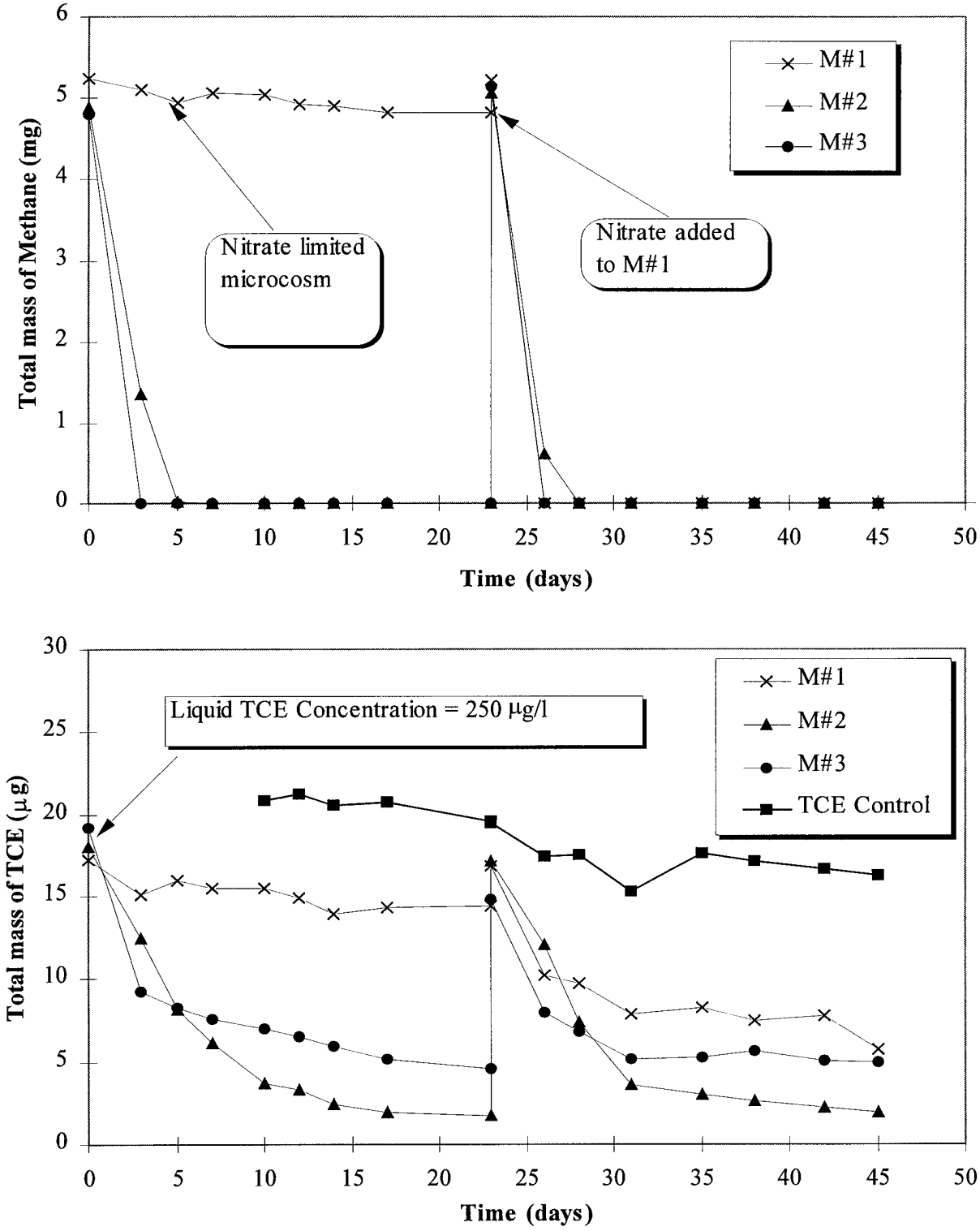


Figure 40. Effects of nitrate addition on methane degradation and TCE transformation.

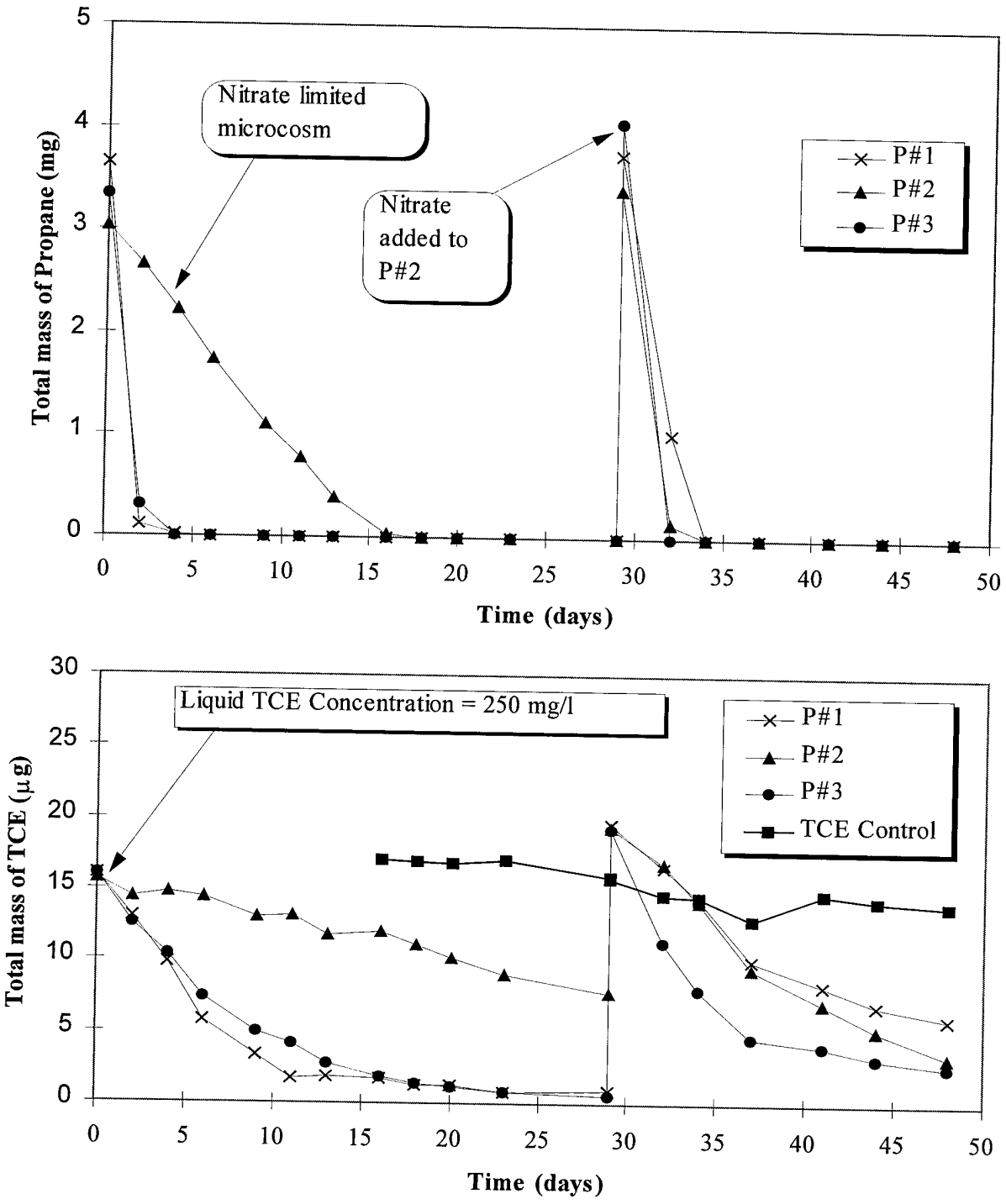


Figure 41. Effects of nitrate addition on propane degradation and TCE transformation.

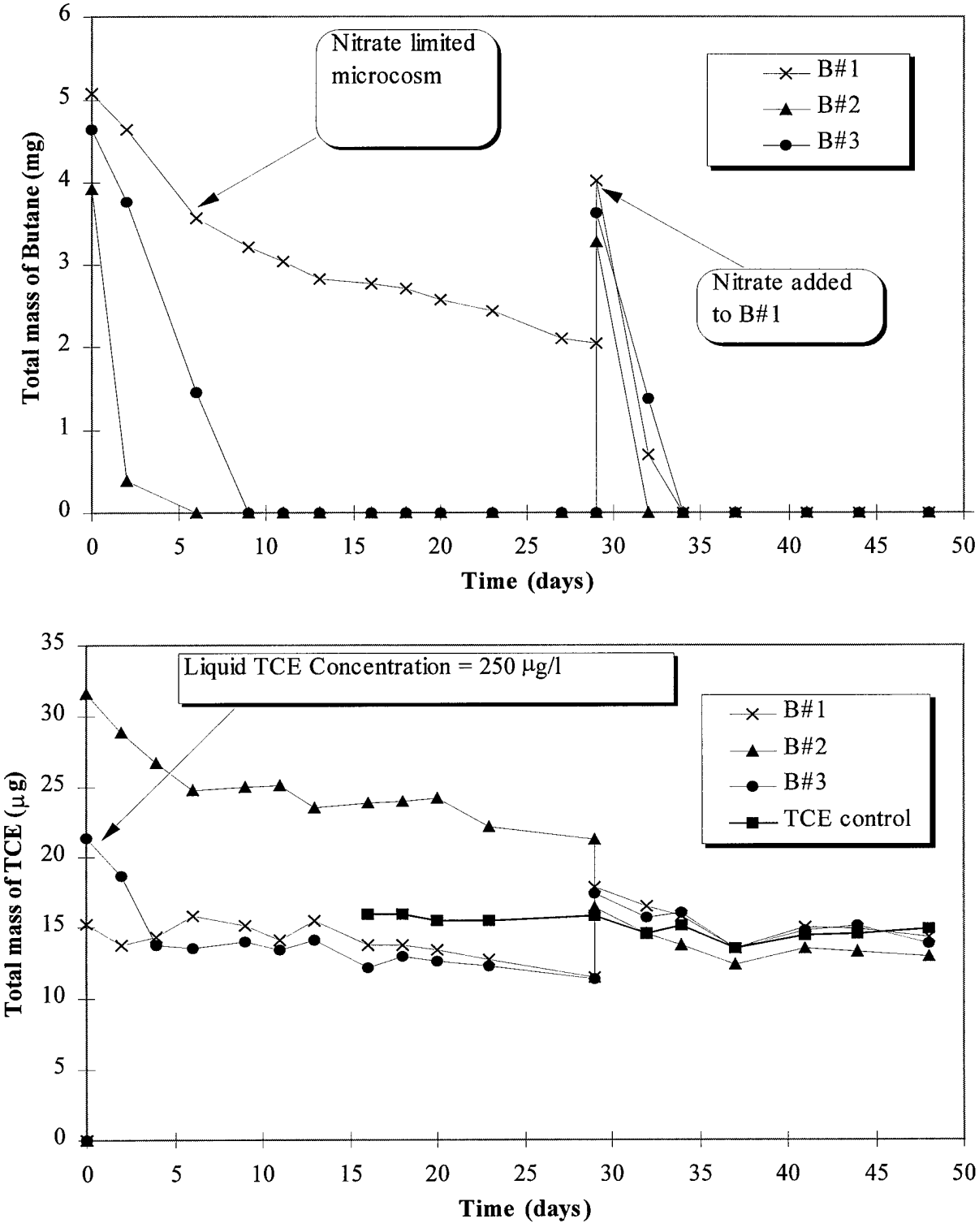


Figure 42. Effects of nitrate addition on butane degradation and TCE transformation.

2. Microcosm studies with methane- and propane-enrichment cultures

Methane and propane enrichment cultures were used to study the effects of nutrient limitations on TCE cometabolism. In the study, successive addition of substrate and TCE were made without exchanging groundwater or media for over 150 days. The study was conducted in batch microcosms without aquifer solid materials present. Figure 43 shows methane degradation and TCE transformation achieved by methane-utilizers after inoculation of 1 ml of a microbial enrichment into the microcosm containing a media formulation previously present. The media that initially contained 0.1 μM of $CuCl_2.2H_2O$ and 23.5 mM of $NaNO_3$ (2000 mg/L of $NaNO_3$) was diluted 1/50 in groundwater. Methane microcosm MM showed an increase in TCE transformation ability over 150 days of incubation without adding or exchanging of media. During the first 60 days of incubation, limited TCE transformation was observed in this microcosm. However, the rate and extent of TCE transformation gradually increased and complete removal of TCE was observed after 75 days of incubation. Competitive inhibition was not observed with increasing of TCE concentration. Population shifts in the microcosm may have occurred resulting in an increase TCE transformation ability. This methane microcosm achieved the highest TCE transformation yield of up to 0.21g TCE/g methane.

Figure 44 and 45 present methane degradation and TCE transformation achieved by methane-utilizers after inoculation of the microcosm with groundwater and 50 % media/ 50% groundwater (MMG), respectively. The nitrate concentration of groundwater microcosm MG was initially 33 mg $NaNO_3$/L, resulting from 1 ml of media being present in the inoculation of batch suspended cultures. The concentration of nitrate in 50% media/50% groundwater was initially 1000 mg $NaNO_3$/L. The results show that during the first 60 days of incubation, complete TCE transformation was observed in both methane microcosms MG and MMG. Methane microcosm MG, containing 100 % GW, was initially the most effective in transforming TCE followed by methane microcosm MMG, containing with 50% media / 50% groundwater. Effective TCE transformation continued with increasing of TCE concentrations. The maximum TCE transformation yields obtained from methane microcosms MG and MMG are less than those of methane microcosm MM. At the end of the 150 days of incubation, nitrate was not present in any of the microcosms.

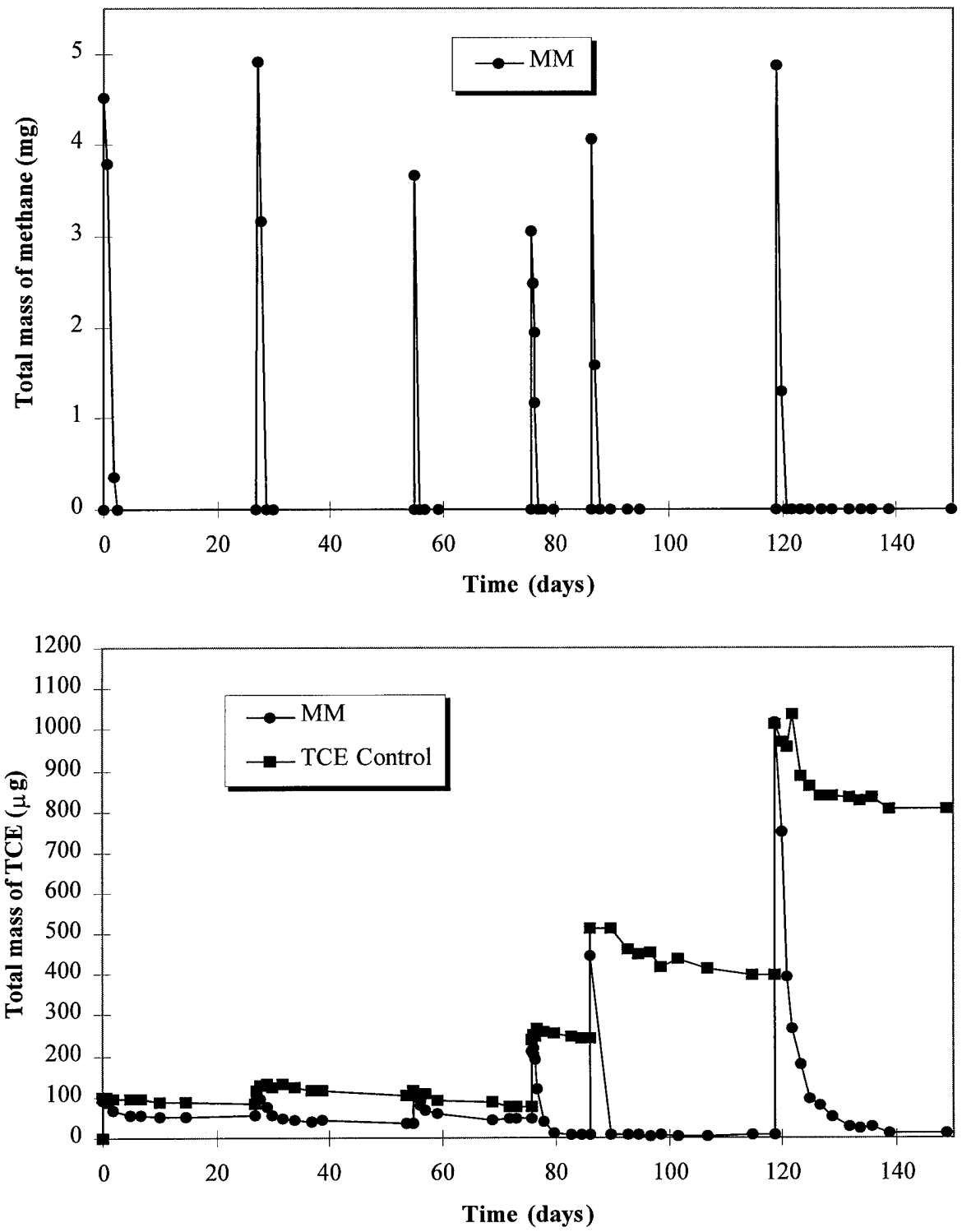


Figure 43. Methane degradation and TCE transformation in the microcosm containing 100% media.

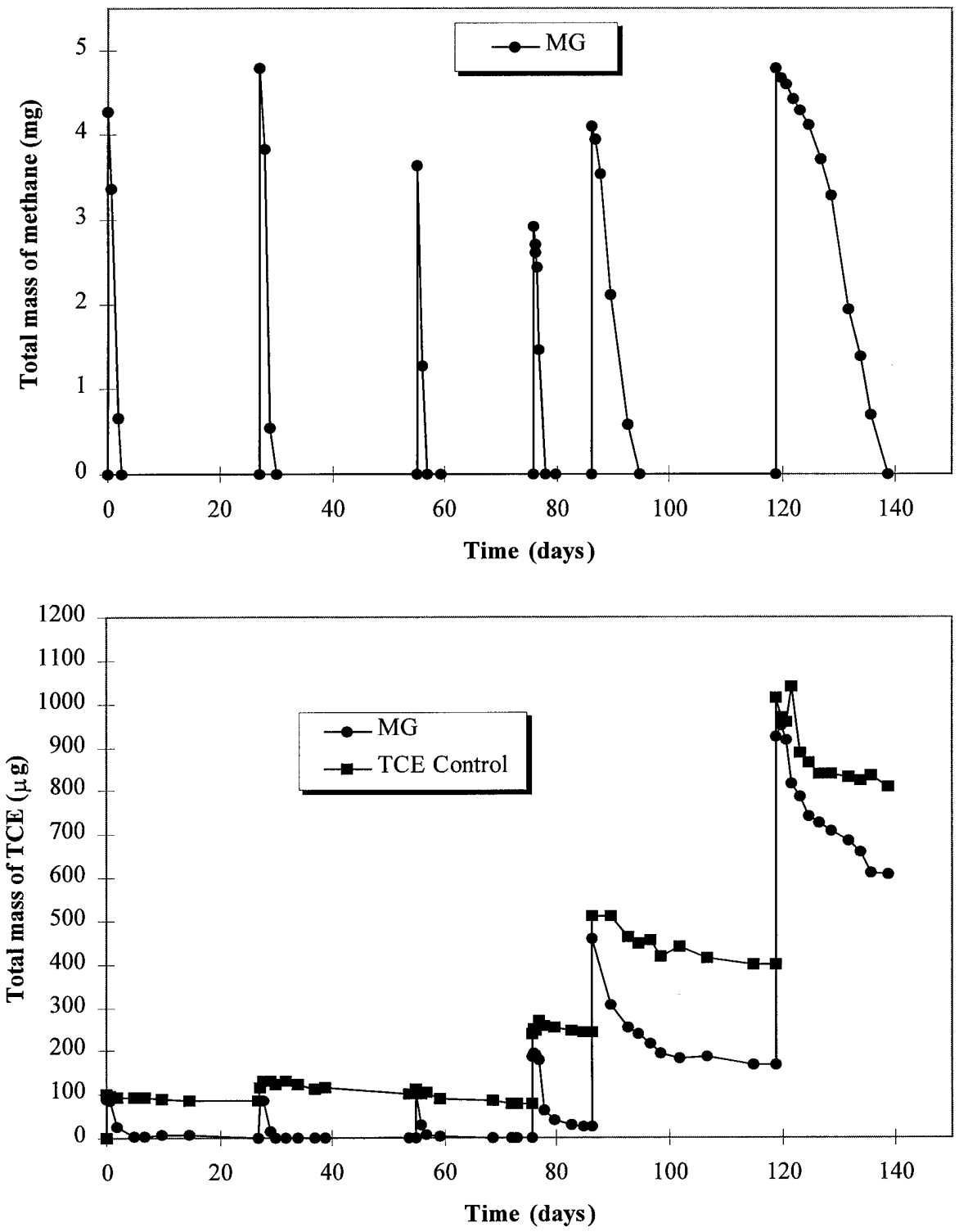


Figure 44. Methane degradation and TCE transformation in the microcosm containing 100% groundwater.

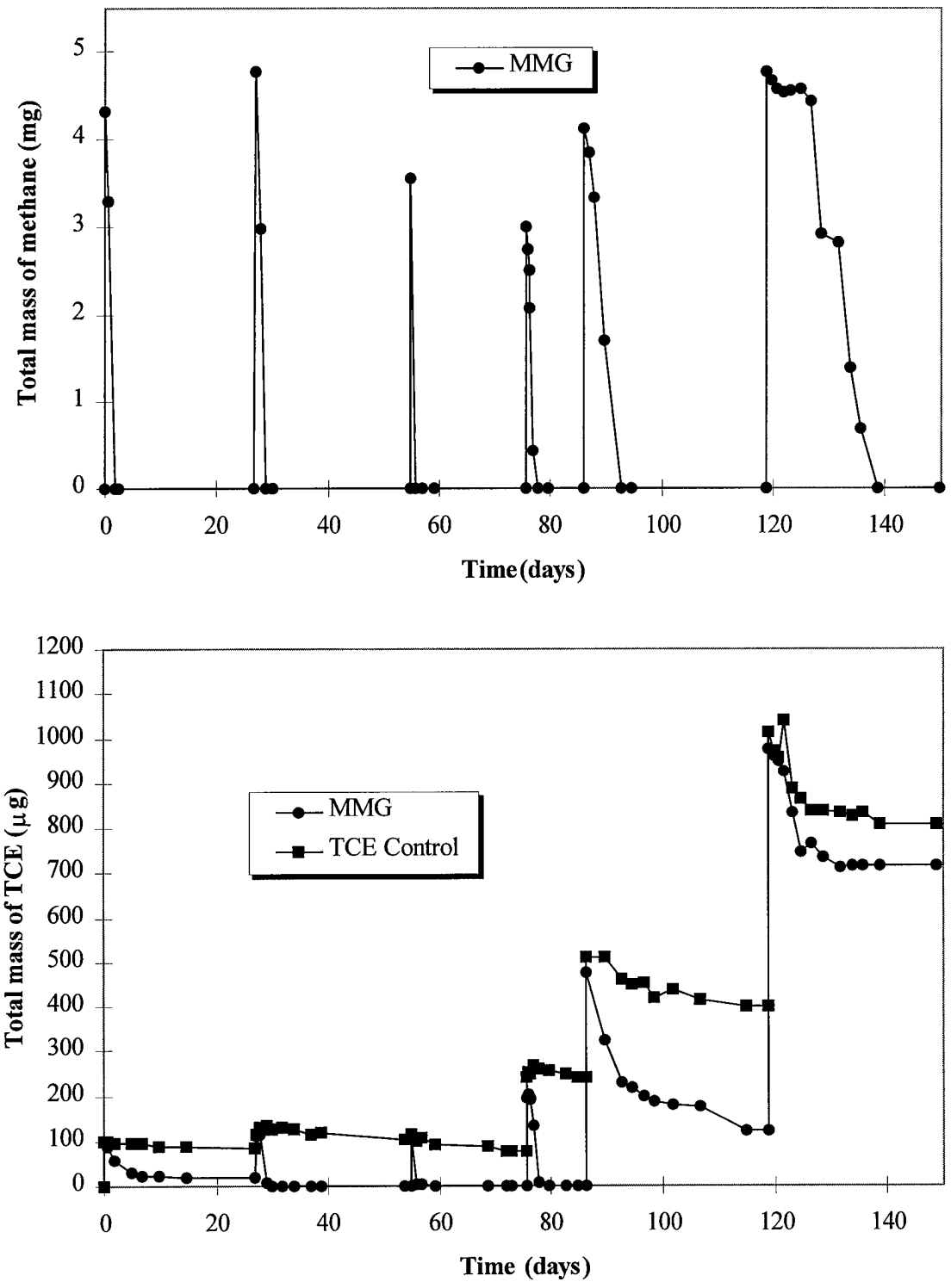


Figure 45. Methane degradation and TCE transformation in the microcosm containing 50/50% (media/groundwater).

Figures 46 and 47 are the results from the control microcosms to which TCE was added after 85 days of incubation in order to confirm the different TCE transformation abilities on media and groundwater. The results show that TCE transformation in media and groundwater control microcosms were similar to those observed in methane microcosms MM and MG, respectively. This confirmed that the microcosms originally having nutrient rich conditions more effectively transformed TCE. The methane results also indicate that earlier exposure to TCE was not causing the differences in the microcosms. As will be discussed, we feel that the improved TCE transformation ability in the methane-utilizers grown in media may have resulted from nitrogen limitations that occurred as time proceeded.

The transformation yields for TCE by methane microcosms containing the different media formulations are presented in Figure 48. All microcosms exhibit an increase in TCE transformation yields with increasing TCE concentrations and incubation time. The early time transformation yields were limited by the mass of TCE present. The microcosms containing 100% media exhibited lower transformation yields during the first 60 days of incubation than the microcosms containing 100% groundwater or 50% media/ 50% groundwater. After 75 days of incubation, the situation was reversed, with the highest TCE transformation yield (0.21 g TCE/g methane) observed in the methane microcosm containing 100% media.

Figure 49, 50, and 51 present the TCE transformation and propane degradation in the propane microcosms with the different media conditions. Unlike methane microcosms, all propane microcosms showed similar TCE transformation abilities over 120 days of incubation. Transformation yields for TCE among propane microcosms were less than those observed in methane microcosms. TCE transformation yields of 0.013 g TCE/ g propane were observed in all propane microcosms, which was less than achieved in the propane microcosms with aquifer solids (0.04 g TCE/g propane). Prolonged TCE transformation activity after propane was consumed was not observed in these propane enrichments. It is interesting that long term TCE transformation activity was achieved with aquifer solids and in the background groundwater chemistry. Thus, the lack of aquifer solids and different chemistry of the microcosms might have affected to TCE transformation abilities.

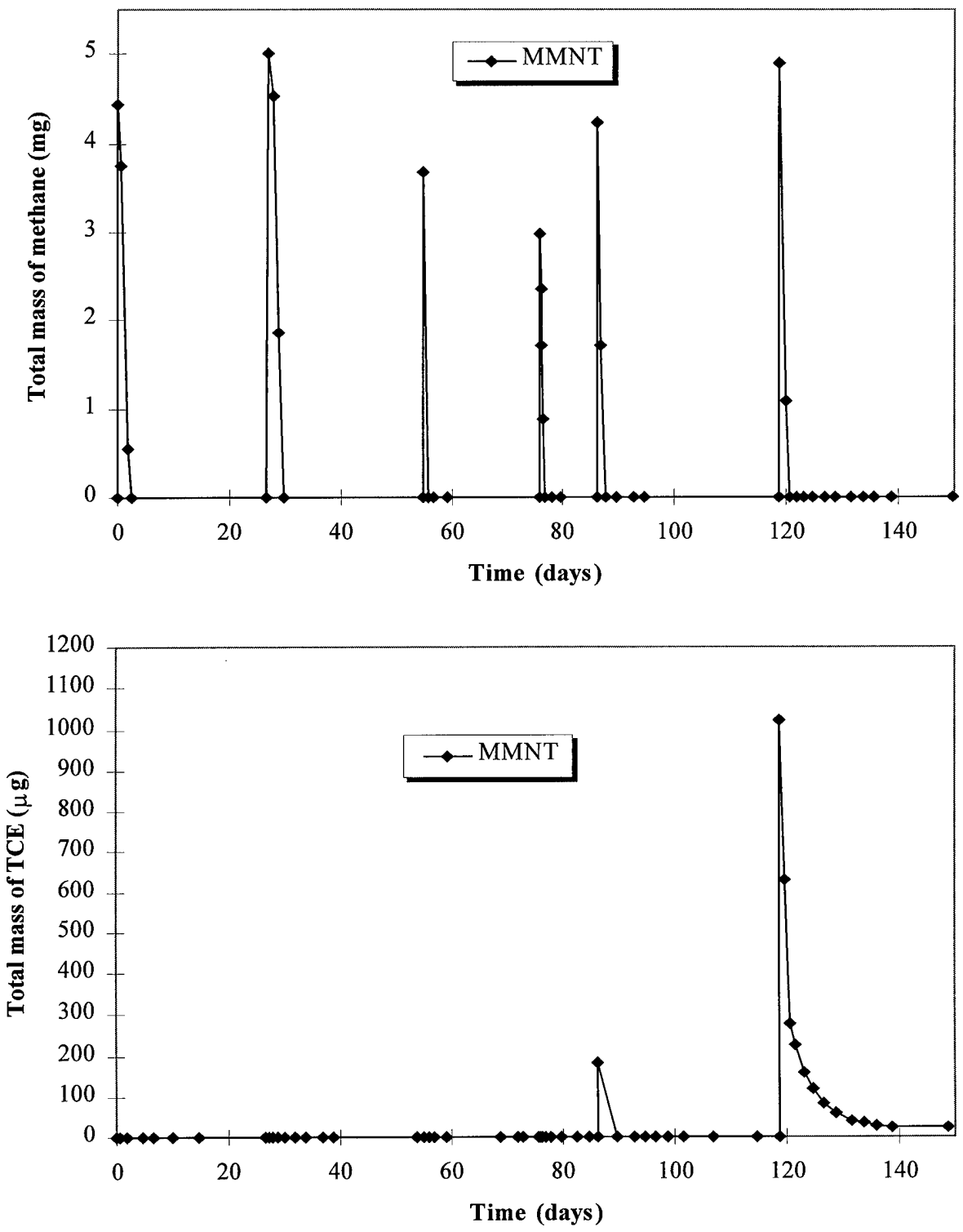


Figure 46. Methane degradation and TCE transformation in the control microcosm containing 100% media.

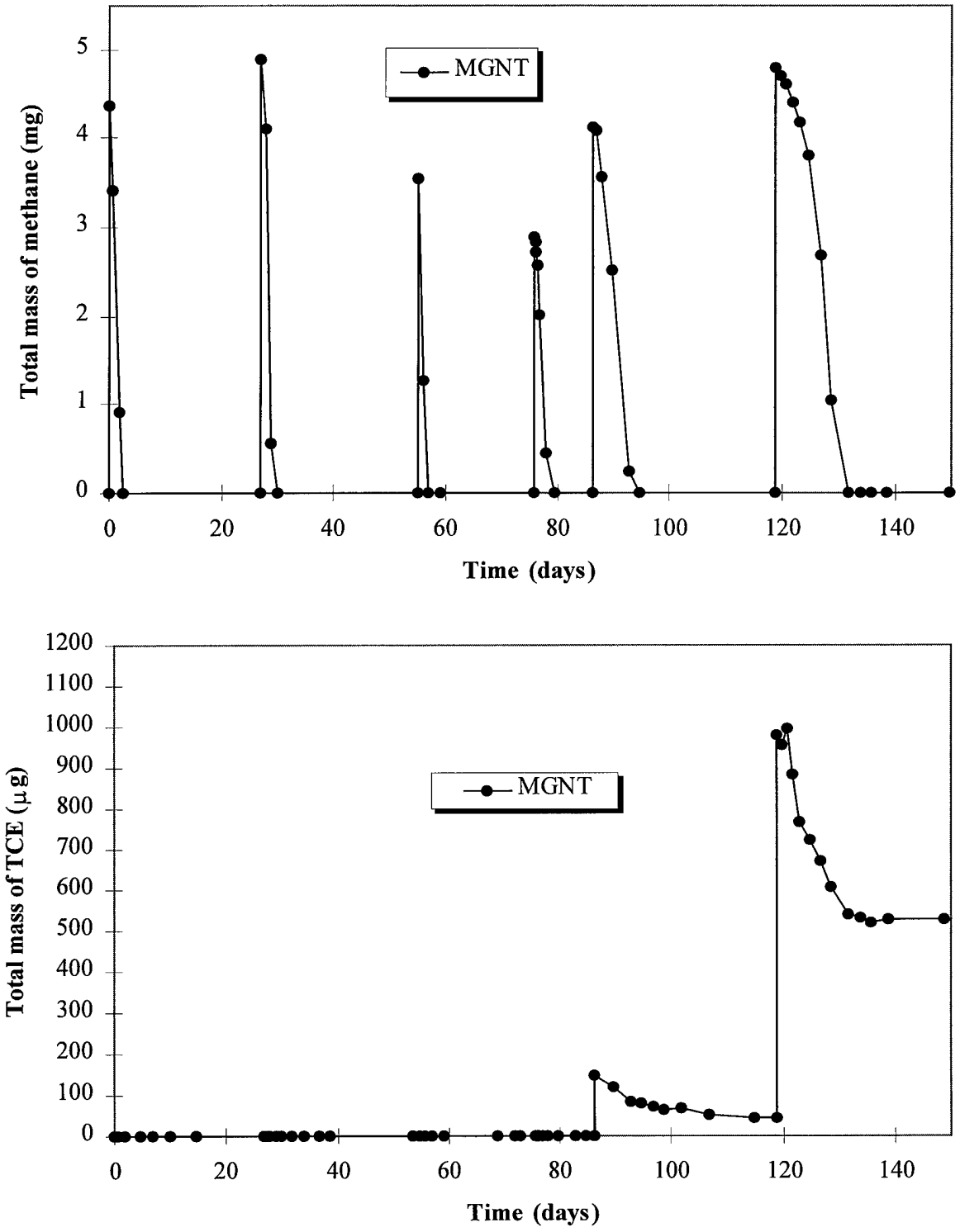


Figure 47. Methane degradation and TCE transformation in the control microcosm containing 100% groundwater.

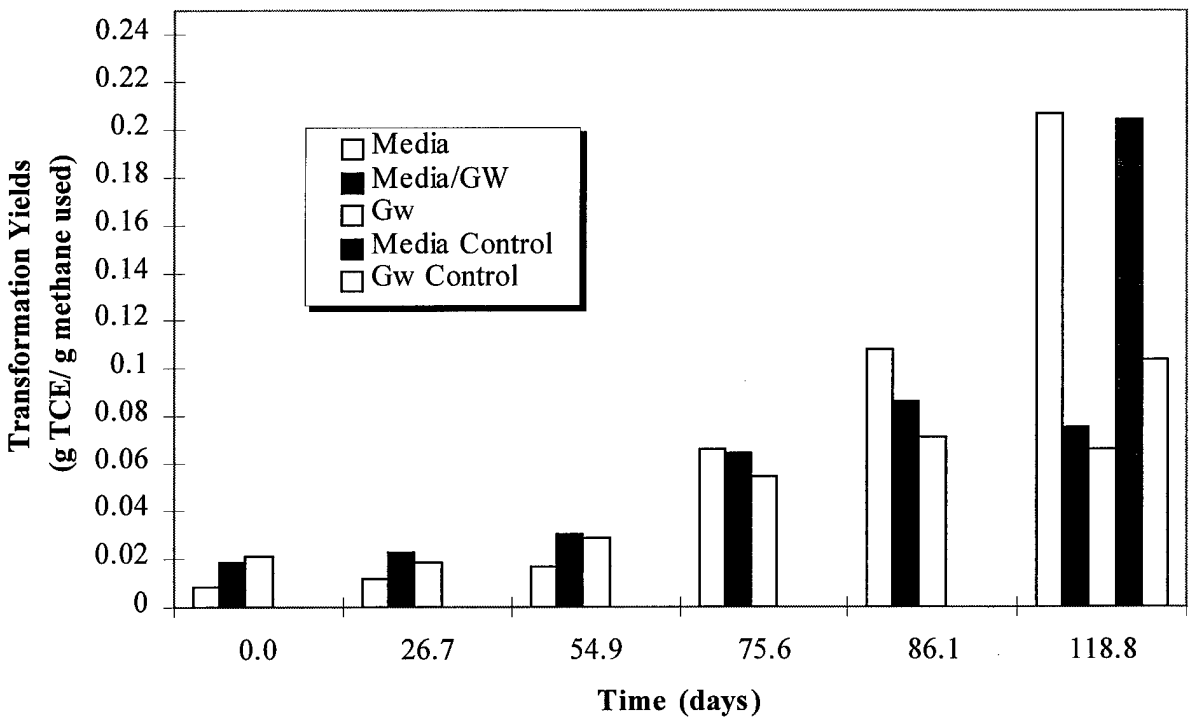


Figure 48. Transformation Yields for TCE by methane-utilizers grown under different medium conditions.

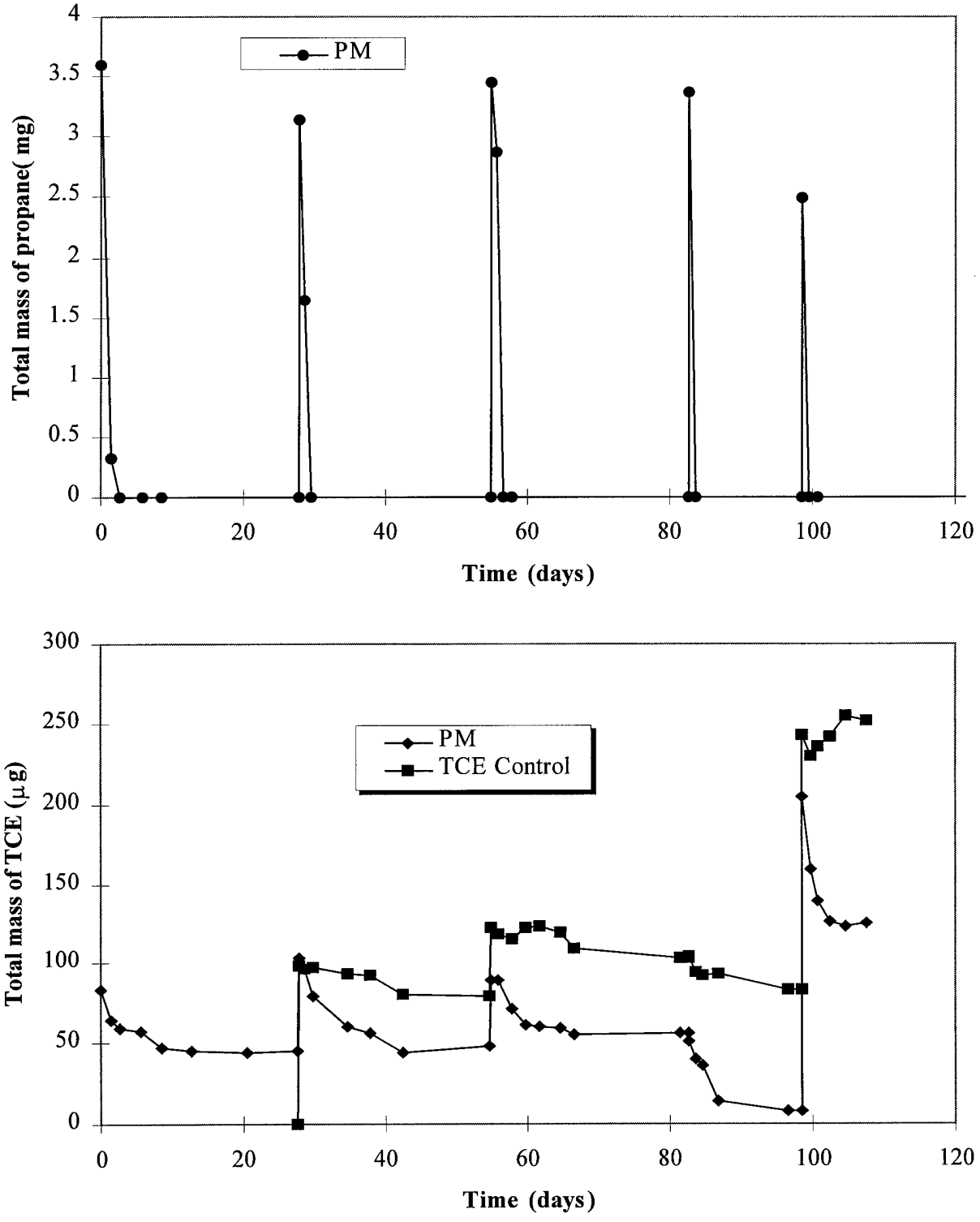


Figure 49. Propane degradation and TCE transformation in the microcosm containing 100% media.

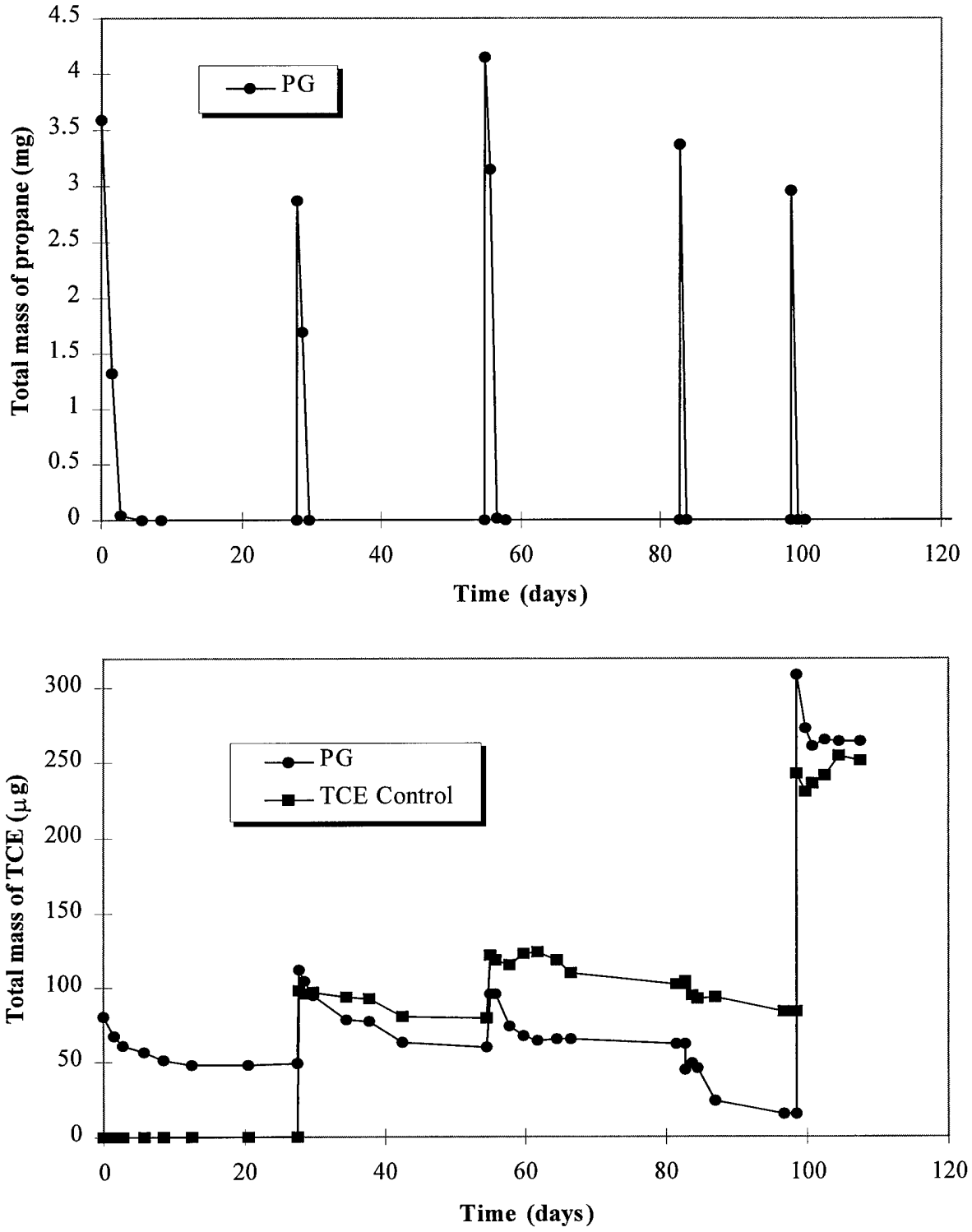


Figure 50. Propane degradation and TCE transformation in the microcosm containing 100% groundwater.

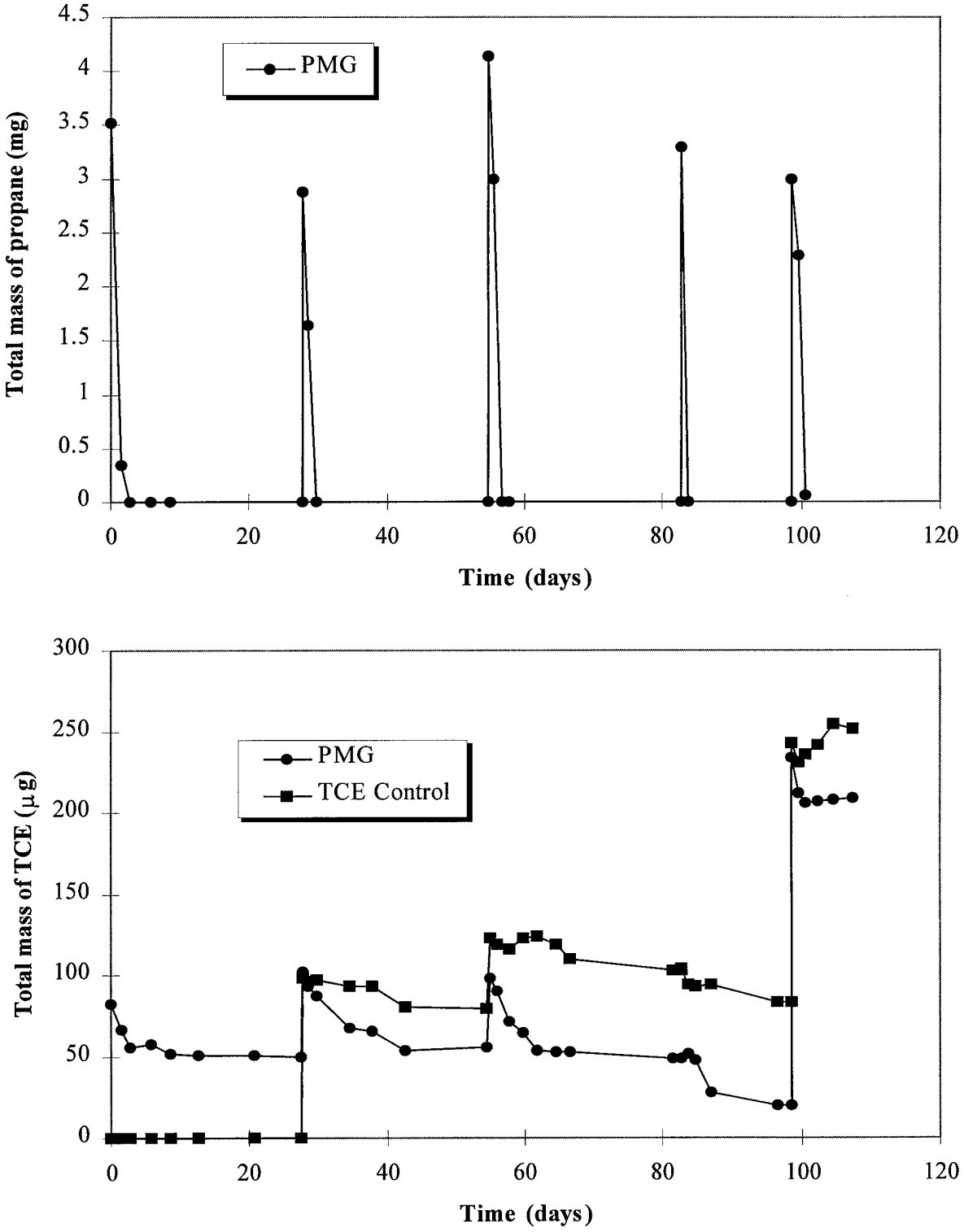


Figure 51. Propane degradation and TCE transformation in the microcosm containing 50/50 % (media and groundwater).

3. Resting cells studies with methane-utilizers grown on different media conditions.

Methane-utilizers stimulated from microcosms (MM, MG, and MMG) were enriched in 750-mL serum bottles under different media conditions to study the effect of nitrate addition on methane degradation. Figure 52 shows the results of growth tests under 6 different media conditions. The most rapid growth was observed on a nutrient rich media without nitrate. The slowest rates of growth were in groundwater with and without nitrate. Rates of growth were somewhat faster when minor nutrients were added to groundwater with and without nitrate present.

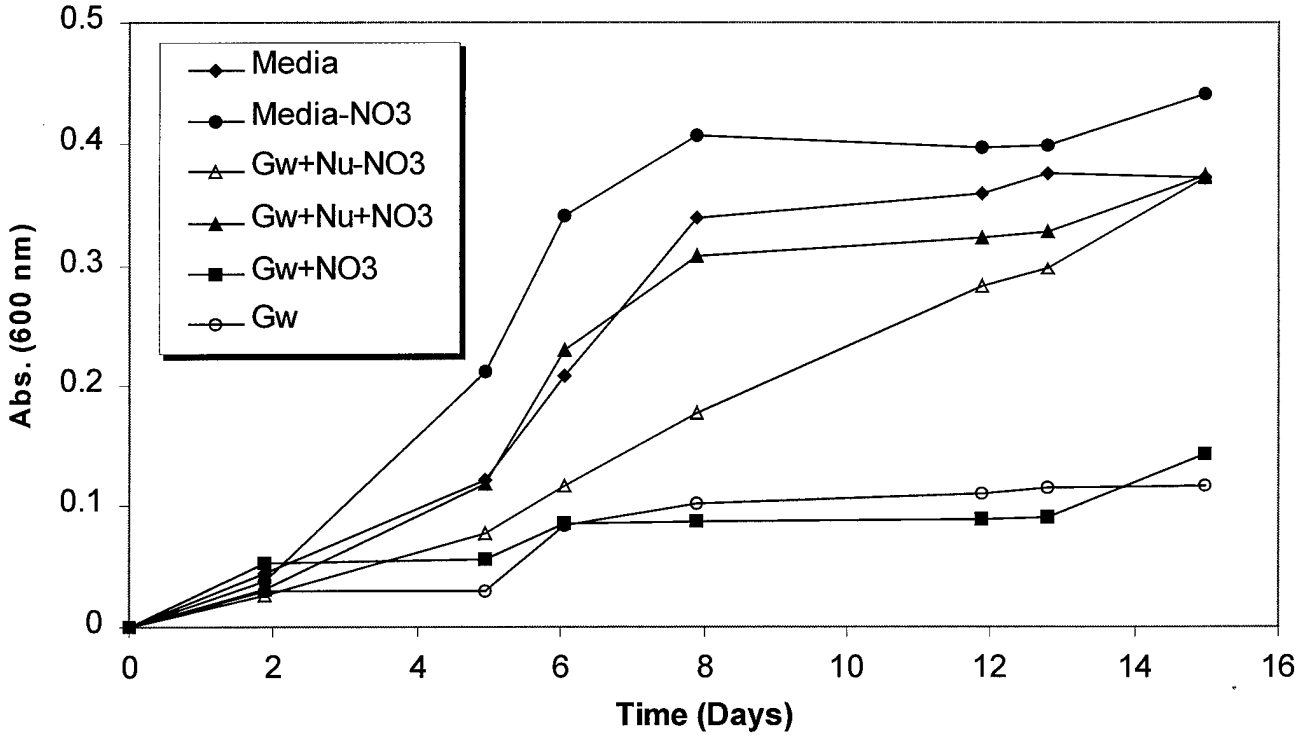


Figure 52. Growth of methane-degrading microorganisms under different media conditions, as measured by optical density at a wavelength of 600 nm. Symbols: ♦, media with nitrate; •, media without nitrate; Δ, groundwater with nutrients lacking nitrate; ▲, groundwater with nutrients or nitrate; ■, groundwater with nitrate; O, groundwater.

Figure 53 presents the utilization of methane by the enrichments grown on different media conditions. The experiment was conducted using 28-mL serum bottles containing with 15 ml of liquid and 13 ml of headspace. 5 mg (dry cells weight) of resting cells was added to the screen bottles. The results indicate that methane-utilizers grown on media or groundwater without nitrate utilized methane faster than those cells grown on media with nitrate.

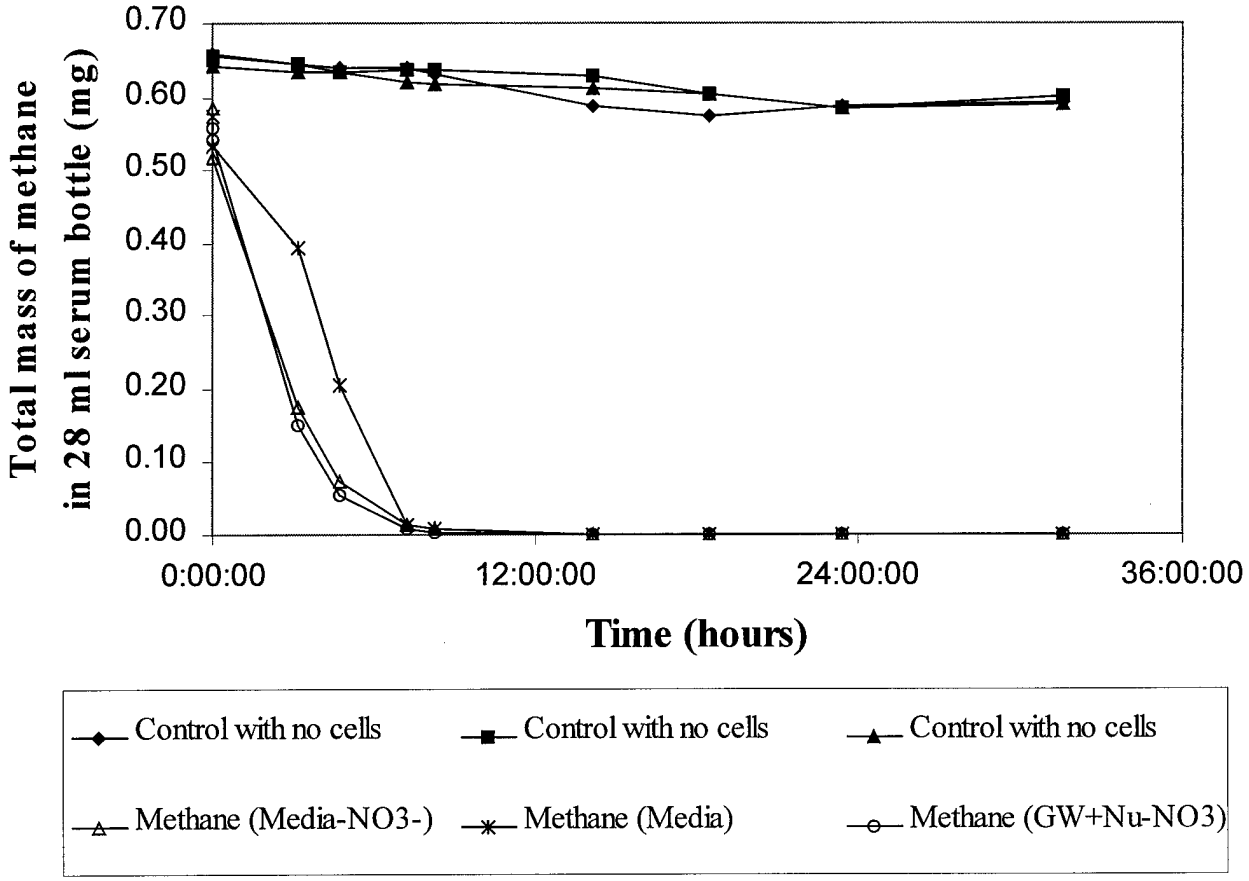


Figure 53. Methane utilization by methane-utilizers grown under different media conditions.

D. DISCUSSION

The results from microcosm studies with aquifer solids and groundwater indicated that the availability of nitrogen (as nitrate) strongly influenced TCE transformation. When nitrate was a limiting nutrient earlier in the incubations, TCE transformation by both methane and propane-utilizers was greatly reduced, even under conditions where the primary substrate was utilized, but at a reduced rate. The results from butane microcosms showed nitrate addition increased butane utilization but not TCE transformation. The addition of nitrate is necessary during biostimulation for effective in-situ cometabolism, when a nitrogen source is limiting in the subsurface.

The results from methane enrichment cultures grown under different medium-conditions show that nutrient availability had a great impact on TCE transformation ability of methane-utilizers. All methane microcosms continued their ability to transform TCE over 150 days without exchanging media or groundwater. TCE transformation yields in all methane-utilizers increased with time, however TCE transformation yields at early time were limited by the mass of TCE present. Nutrient availability, possibly nitrate or copper, may have played major roles on methane-utilizer's ability to transform TCE. Low copper concentration (0.1 μM) in the microcosms established the copper-limited conditions that may be favorable to the expression of sMMO. These results were consistent with previous study by Graham et al., 1993. They revealed that copper limitations favored Type II *M. trichosporium* OB3b. The Type II organisms were dominated under conditions that induced sMMO expression. The sMMO activity appeared at a measurable soluble copper concentration level of 0.15 μM.

We also suspect that nitrogen fixation occurred in the microcosms resulting in an increase TCE transformation ability. Depletion of nitrate in the microcosms presumably resulted in nitrogenase activity. Type II methanotrophs (sMMO form) which were capable of fixing nitrogen may have been selected when nitrate was limited in the microcosms. These results are consistent with the prior studies. Type II methanotrophs that efficiently transformed TCE accumulated high PHB contents under copper and nitrogen-limited conditions (Graham et al., 1993; Chu and Alvarez-Cohen, 1996; Shah et al., 1996). It is also interesting that our maximum TCE transformation yield of 0.21 (g TCE/g methane) observed in methane microcosms upon depletion of nitrate were higher than the prior studies with resting cells of nitrogen-fixing methanotrophs. TCE transformation yields of 0.020, and 0.11 (g TCE/ g methane) (0.25 g TCE/ g dry cell wt, assuming net cell yield of 0.4 g dry cell wt / g methane), were observed by Chu and Alvarez-Cohen, 1996 and Shah et al., 1995, respectively. This study suggests that TCE transformation may be enhanced by methane-utilizers for in situ bioremediation by first growing them under rich nutrient conditions and then limiting nutrients such as nitrogen. Further research is required to test this hypothesis.

SECTION VI

COMETABOLISM OF TCE AND 1,1,1-TCA BY MICROBES GROWN ON MIXED COMETABOLIC SUBSTRATES: METHANE AND PROPANE OR PHENOL AND PROPANE

A. INTRODUCTION

Chlorinated aliphatic hydrocarbons (CAHs) such as 1,1,1 TCA and TCE are among the most widespread contaminants in groundwater and soil. Both compounds were widely used as industrial solvents and military extraction agents. 1,1,1 TCA has also been detected along with TCE in the groundwater and subsurface of the McClellan AFB. 1,1,1 TCA also can be abiotically converted to 1,1-DCE in the subsurface (Vogel and McCarty, 1987). 1,1,1- TCA, 1,1-DCE and TCE are of particular concern due to their toxicity and carcinogenicity. They are regulated by EPA to a maximum contaminant level of 0.005 mg TCE/L, 0.2 mg 1,1,1-TCA/L, and 0.005 mg 1,1-DCE/L (Cook, 1987; McCarty and Semprini, 1994).

Under aerobic conditions, many chlorinated hydrocarbons can be cometabolically transformed by microorganisms grown on methane (Wilson et al., 1985), propane (Wackett et al., 1989), phenol (Nelson et al., 1987), and toluene (Nelson et al., 1987; Wackett et al., 1988). The selection of suitable microorganisms to transform specific CAHs in soil and groundwater are of interest for in situ bioremediation. Microorganisms, that catalyze the transformation of a significantly broad range of contaminant substrates, are desirable for enhancing in situ bioremediation, since groundwater and soil are commonly contaminated with multiple chlorinated hydrocarbons.

Methane-utilizing bacteria have been reported to transform a broad range of chlorinated hydrocarbons including TCE and 1,1,1 TCA (Fogel et al., 1986; Chang and Alvarez-Cohen, 1994 and 1996; van Hylckama Vlieg et al., 1996; Little et al., 1988; Oldenhuis et al., 1989 and 1991). Among TCE and 1,1,1-TCA, methane-utilizing cultures in resting cell studies exhibited highest transformation capacity for TCE, follows by 1,1,1- TCA (Change and Alvarez-Cohen, 1996).

Heterotrophic bacteria grown on phenol and toluene were able to transform TCE, but not saturated compounds such as TCA (Wackett et al., 1988; Nelson et al., 1986; Fliermans et al., 1988; Chang and Alvarez-Cohen, 1994). Resting cell studies have also shown that microorganisms grown on propane were able to transform both compounds, but with transformation capacity values less than those of methane-utilizers (Chang and Alvarez-Cohen, 1994). The transformation and inhibition of TCE (Wackett et al., 1989) and 1,1,1 TCA (Keenan et al., 1993) by propane has been observed by propane-oxidizing cultures. They demonstrated the propane-monooxygenase enzyme was responsible for the transformation kinetics and inhibition of TCE and 1,1,1 TCA cometabolism. Moreover, the propane oxygenase enzyme, which is responsible for initial oxidation of propane, is

nonspecific enough to metabolize and oxidize short-chain alkenes and other aliphatic hydrocarbons (Hou et al., 1983; Perry, 1980).

Previous research has shown that CAHs, such as TCE exerts transformation product toxicity on methane-utilizers, decreasing their ability to transform CAHs (Broholm et al., 1990; Alvarez-Cohen and McCarty, 1991; Speitel et al., 1993; Oldenhuis et al., 1989 and 1991). The presence of 1,1,1-TCA in the groundwater is of concern, because it can impact TCE transformation. Competitive inhibition among growth substrates and the CAHs can decrease the rate of CAH transformation (Oldenhuis et al., 1991). Toxicity resulting from CAH transformation also causes inactivation and limited transformation capacity (Alvarez-Cohen and McCarty, 1991).

Developing cometabolic systems that effectively transformed TCE and 1,1,1-TCA mixtures is therefore of interest. Based on our observations with microcosms from McClellan AFB, discussed in Section IV, mixtures of methane and propane might be of interest to transform a broader range of compounds including TCE and 1,1,1-TCA. Phenol and propane might also be of interest as mixed-cometabolic substrates for transformation of TCE and 1,1,1-TCA.

The goal of this phase of the research was to determine whether more effective transformation of CAH mixtures could be achieved by stimulating microbes on mixed cometabolic substrates. Two pairs of mixed-cometabolic substrates were evaluated: 1) methane and propane; and 2) phenol and propane. TCE and 1,1,1 TCA were selected as the chlorinated hydrocarbons of interest because these compounds are observed in the subsurface of McClellan AFB. Methane and phenol were chosen as the cometabolic substrates to promote effective transformation of TCE. Propane was chosen as a good substrate to promote the transformation of 1,1,1-TCA. Also propane showed long term transformation abilities that might prove useful. We also decided to focus on different cometabolic systems. The methane/propane system represents gaseous-aliphatic cometabolic substrates, while the propane/phenol system represents aliphatic/aromatic substrates.

B. MATERIALS AND METHODS

1. Enrichment of methane and propane-utilizers with media

Mixed methane and propane enrichment cultures used in this study were obtained from batch McClellan microcosm studies presented in Section IV. The methane and propane enrichment cultures, which are capable of TCE transformation, were grown separately in growth medium containing with: 15 mM, K_2HPO_4 + NaH_2PO_4 (a buffer 7.5 solution), 0.5 mM, $MgSO_4$, 0.1 mM, $CaCl_2$, 23.5 mM, $NaNO_3$, 0.796 mM, $(NH_4)_2SO_4$, and 1-mL of trace element solution. The suspended cultures were grown in 500-mL serum bottles at 30 °C on a shaker table at 200 rpm. Each 500-mL serum bottle was supplied with oxygen, methane or propane at 10% by volume in the headspace.

The enrichment cultures were used to study TCE and 1,1,1-TCA transformation. The 125 ml serum bottles were inoculated with 1 ml each of washed suspended cells before the addition of growth substrate and CAHs. The serum bottles contained 60 ml of media and 65 ml of headspace. The serum bottles were crimp sealed with a Teflon™ butyl rubber (Kimble Co., IL), inverted and incubated at room temperature on a shaker table at 140 rpm. Pulsing and mixing strategies of methane and propane were used to study the effect of methane or/and propane on TCE and TCA transformation.

2. Indigenous phenol and propane microcosm studies with aquifer solids

For these studies, batch microcosms constructed with aquifer solids and groundwater from McClellan Air Force Base (as described in Section IV) were used. The tests were performed with an experimental design using fourteen microcosms (Table 14). Propane or phenol were evaluated as single substrates, PR1 and PH1, respectively. Dual-substrate pulsing of propane and phenol was used in all other microcosms, except microcosms PR1 and PH1. Microcosms PR6 and PH6 are the propane and phenol control microcosms, respectively, that lacked CAH addition. CAH-control microcosms (PR7 and PH7) contained TCE and 1,1,1-TCA, but lacked the cometabolic growth substrate. The microcosm series were constructed to compare the performance of the mixed substrates to single substrates. We also tested whether stimulating indigenous microbes with phenol first and then switching to propane, or propane first and switching to phenol made any difference on the microcosm performance. We also determined whether difference efficiencies resulted when the microbes were challenged with CAH mixtures compared to single CAH (TCE or 1,1,1-TCA).

Table 14. Phenol and propane fed microcosms used in the study

Microcosms		Initial substrate fed	Containing with TCE	Containing with TCA
Phenol	PH1	Phenol	TCE	TCA
	PH2	Phenol	TCE	TCA
	PH3	Phenol	TCE	TCA
	PH4	Phenol	TCE	-
	PH5	Phenol	-	TCA
	PH6	Phenol	-	-
	PH7	CAH Control	TCE	TCA
Propane	PR1	Propane	TCE	TCA
	PR2	Propane	TCE	TCA
	PR3	Propane	TCE	TCA
	PR4	Propane	TCE	-
	PR5	Propane	-	TCA
	PR6	Propane	-	-
	PR7	CAH Control	TCE	TCA

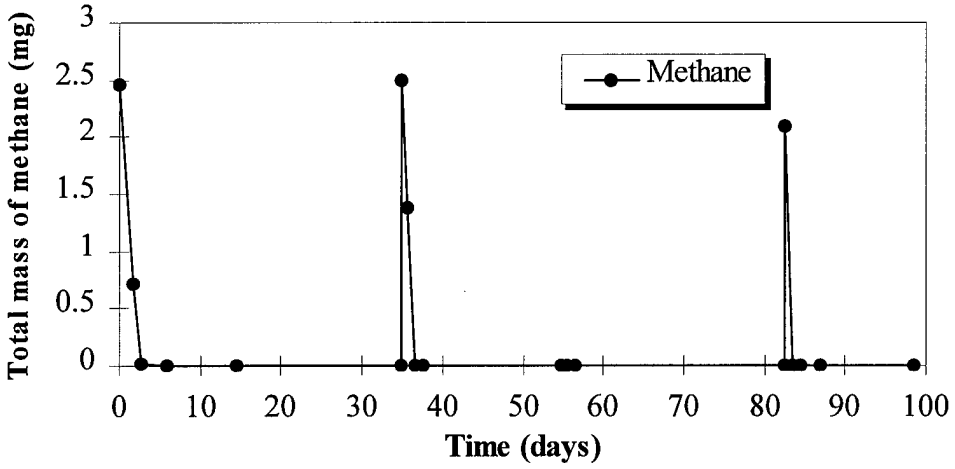
3. Groundwater Amendments

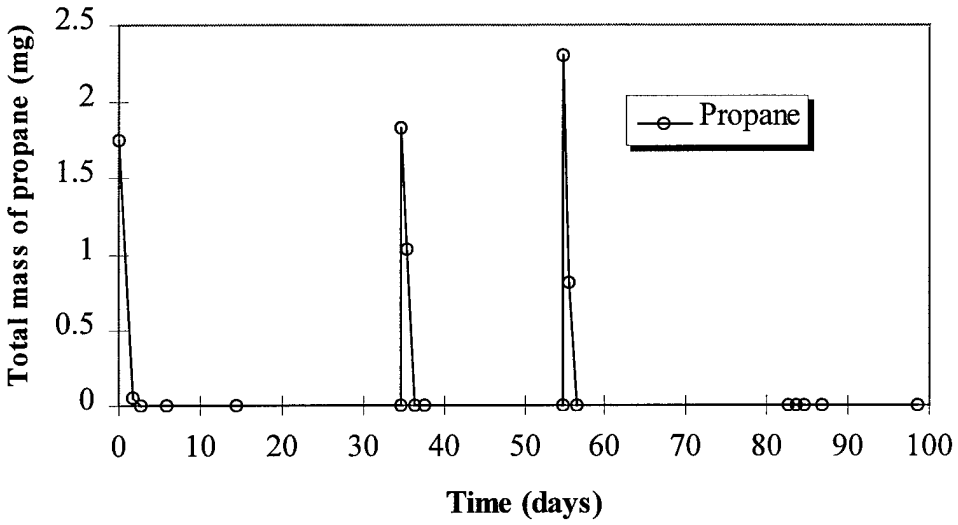
The microcosms were maintained at room temperature with periodic exchange of groundwater and additions of substrate. Groundwater was exchanged prior to the addition of the growth substrate and CAHs. The groundwater was amended with nitrate to 30 mg/L, since nitrogen was found to be limiting in the groundwater. The microcosm operation, sampling, and the analytical procedures are described in Section IV.

C. RESULTS

1. Transformation of TCE and 1,1,1-TCA by methane- and propane-utilizers in media microcosms.

Figure 54 presents the combined stimulation of methane- and propane-utilizers and the resulting TCE cometabolism. Both the uptake of propane and methane was rapid and occurred within several days with each of stimulation, and TCE transformation was observed. When only a single substrate was fed to the microcosm, methane was found to be more effective than propane for TCE transformation. The results indicate that most of the transformation was promoted by the methane-utilizers, when both substrates were being fed. The results are consisted with those reported in Section V for studies with media fed microcosms (MM) and (PM), which were the parent cultures used in these mixed-substrate tests.





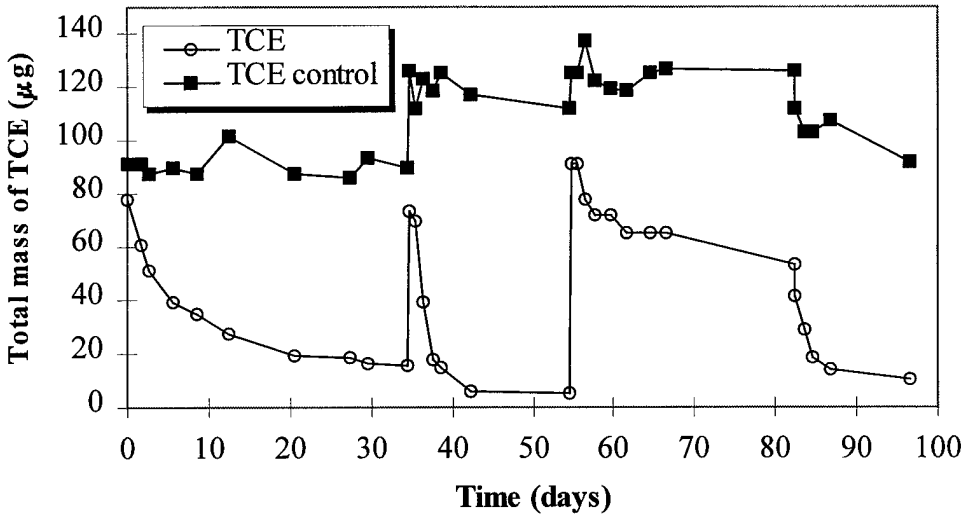
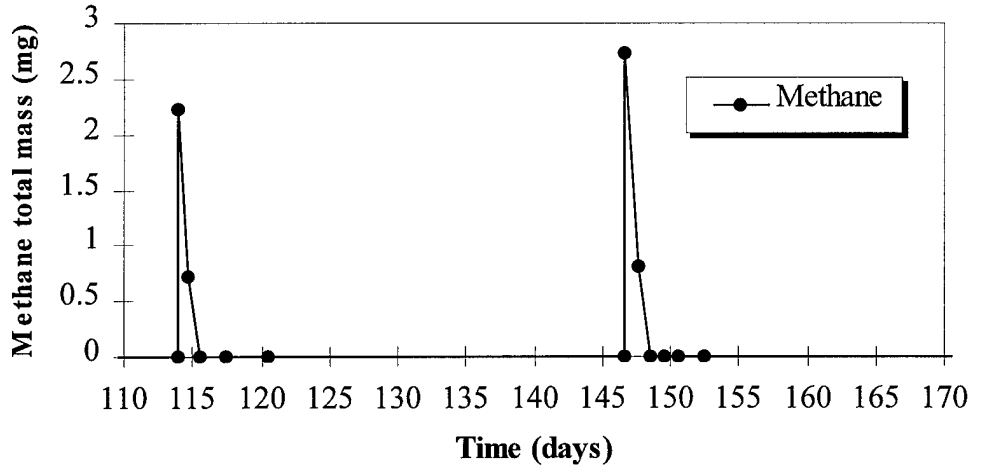


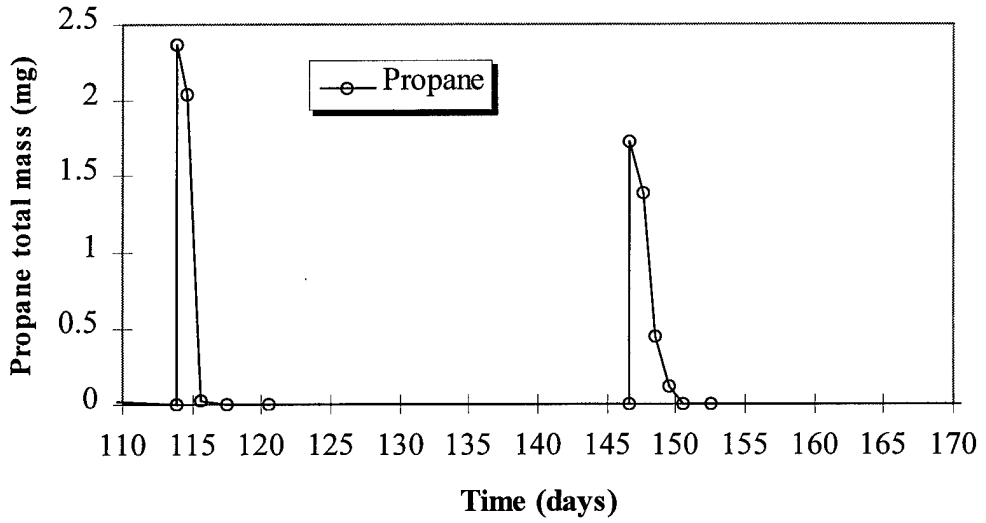
Figure 54. TCE transformation by methane- and propane-fed microcosms during 100 days of incubation

The mixed-substrates effectively transformed TCE, as the TCE concentration was increased (Figure 55). The incubation, starting at 147 days in both propane-and methane-fed microcosms, resulted incomplete TCE transformation. A transformation yield of 0.133 mg TCE/mg substrate was achieved during this period, which is lower than the value of 0.20 mg TCE/mg methane achieved by the methane-utilizers under media conditions (Figure 46, Section V). The results indicate that no distinct advantage of using methane and propane as dual substrates for the cometabolism of TCE, compared to using methane as a single substrate. The results from the single substrate test are consistent with the mixed substrate test, indicating that the methane-utilizers were responsible for most of the TCE transformation observed in the mixed substrate case.

Figure 56 presents one set of microcosm results evaluating the transformation of a mixture of TCE and 1,1,1-TCA using propane and methane as mixed-cometabolic substrates. The initial stimulation with methane showed little transformation of TCE or 1,1,1-TCA despite active methane utilization. When both methane and propane were fed, both TCE and 1,1,1-TCA were transformed. Methane and propane uptake, however, appeared to be inhibited, with methane utilization occurring after propane was mostly consumed and after 1,1,1-TCA was reduced to low concentrations. One possible explanation is that methane utilization was inhibited by the presence of 1,1,1-TCA. Once TCA was decreased to a low level via propane cometabolism, methane utilization started and more TCE was transformed. An interesting observation is that the presence of methane did not inhibit the ability of the propane-utilizers to transform 1,1,1-TCA. The results suggest that methane does not strongly bind to the PMO, and does not interfere with the cometabolism of the CAH. This is important for strategies that may be used for dual substrate addition. More studies are necessary to evaluate these interactions in more detail. The final addition with propane, showed effective 1,1,1-TCA removal and some TCE transformed, which was consistent to our previous work.

The results from the studies with methane and propane as dual-cometabolic substrates indicate that some advantage might be gained through mixed substrate addition. The work indicates that methane and propane-utilizers can be grown together, and they do not strongly inhibit each other. Transformation of 1,1,1-TCA by propane-utilizers may have removed inhibitory effect on methane-utilizers. More work is needed to determine the advantages of this concept, compared to using a single substrate. Propane, for example, can transform both 1,1,1-TCA and TCE. Thus, future work should include a more detailed comparison of this mixed substrate combination to that of the single substrates. Oxygen consumption differences would be of interest, since the ability to deliver oxygen to the subsurface is an important consideration. Also in this work, the long term TCE transformation ability with propane-utilizers, observed in the soil cultures, appeared to be lost in these media enrichments. More effective treatment of the mixtures might have been achieved if this long-term activity had been maintained.





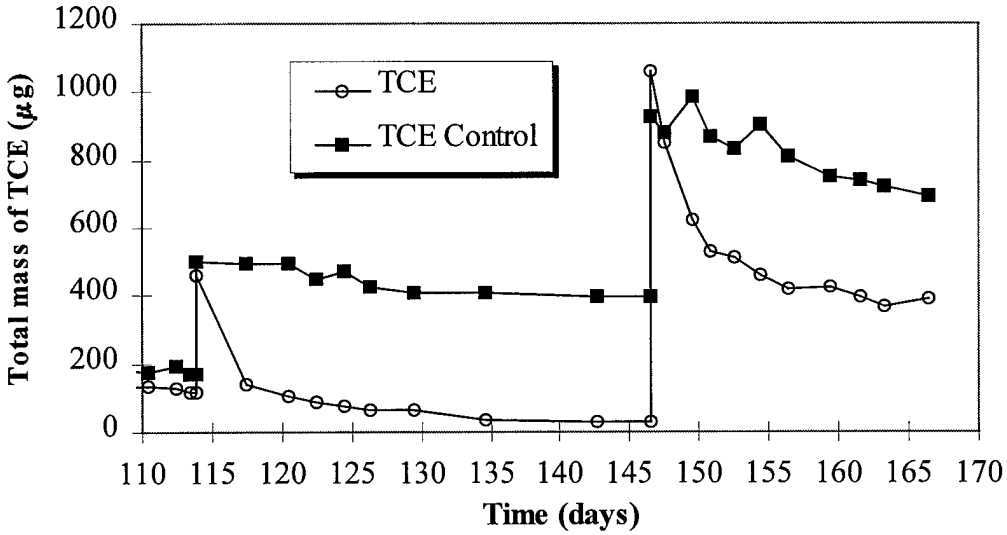
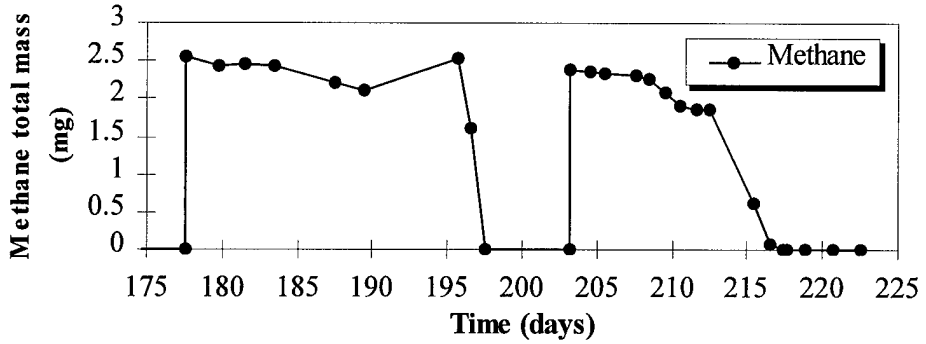
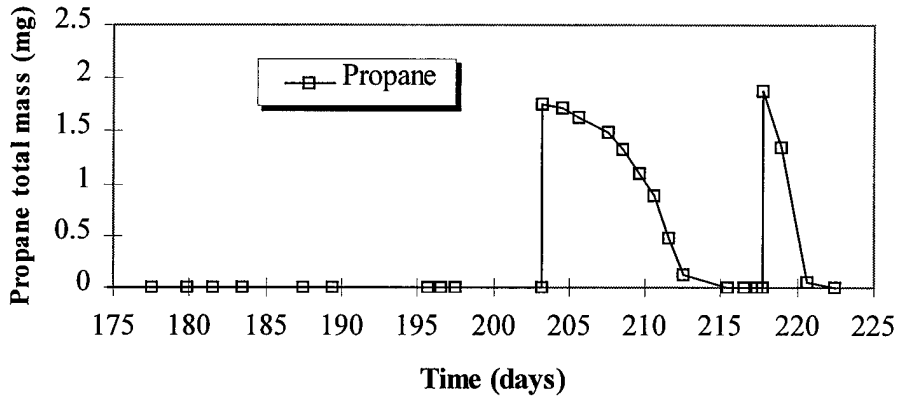
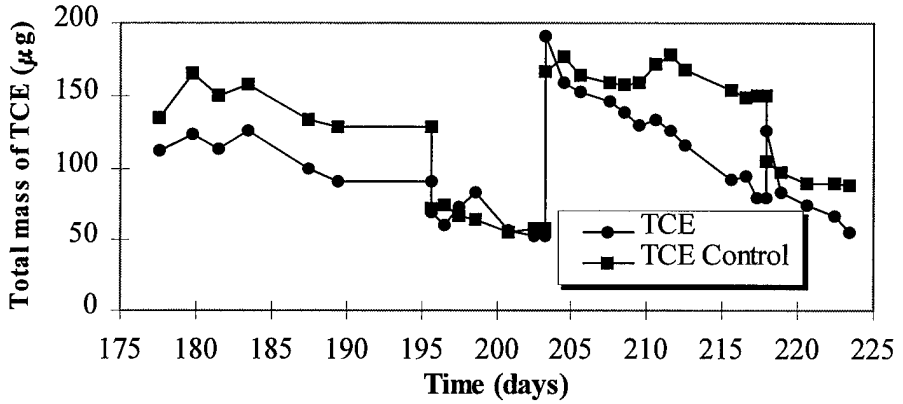


Figure 55. TCE transformation by methane- and propane-fed microcosms with increasing TCE concentrations







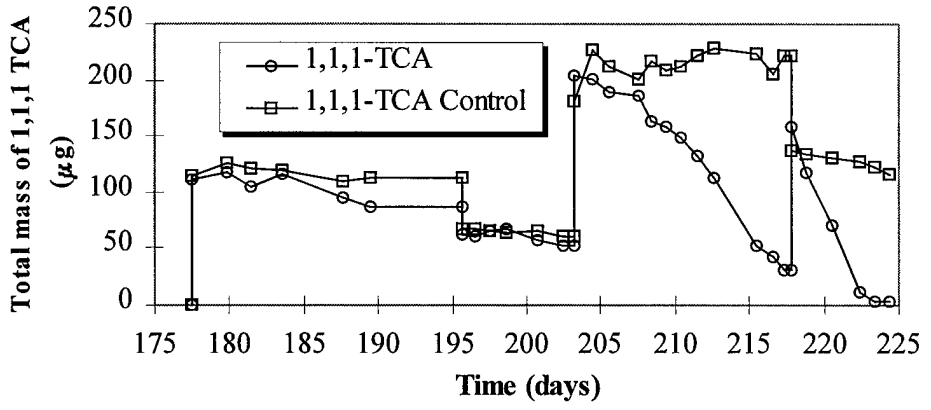


Figure 56. TCE and 1,1,1-TCA transformation by methane- and propane-fed microcosms

2. Transformation of TCE and 1,1,1-TCA in microcosm-fed phenol and propane

The studies with phenol and propane as mixed-cometabolic substrates were performed in soil microcosms constructed with McClellan's aquifer solids and groundwater. The results with the mixed-substrate studies, using methane and propane, showed that the long-term activity observed in the microcosms was not maintained in the media fed system. Since maintaining long-term transformation ability is likely to result in better transformation, we chose to use soil microcosms for these tests. The use of soil microcosms also permits a direct comparison with results presented in Section IV.

Figure 57 presents the results from the tests when propane was fed alone with both TCE and TCA present. Very effective transformation of 1,1,1-TCA was achieved with limited transformation of TCE. 1,1,1-TCA was so effectively transformed that several spikes of 1,1,1-TCA could be degraded upon the consumption of a single pulse of propane, as indicated by the results obtained after 60 days of operation. CAH transformation remained active for periods of 20 days after propane was consumed. Note that slow rates of TCE transformation continue during the periods of rapid 1,1,1-TCA transformation. Thus long-term transformation activity was achieved with propane-utilizers consistent with the results presented in Section IV.

Figure 58 presents the results from the tests when phenol alone was fed to a microcosm with 1,1,1-TCA and TCE present. TCE was transformed effectively by the phenol-utilizing microorganisms, while 1,1,1-TCA was not transformed. The phenol-utilizers remain active towards TCE transformation for short periods after phenol was consumed. The results with the single substrate indicate that effective transformation of CAH mixtures of 1,1,1-TCA and TCE might be achieved through the use of mixed-cometabolic substrates. Phenol would be effective towards TCE transformation, while propane would be effective towards 1,1,1-TCA transformation.

Figures 59 shows the results for 1,1,1-TCA transformation alone when phenol and propane are being alternately pulse fed. When the microcosm was first stimulated on phenol, no ability to transform 1,1,1-TCA was observed. Upon switching to propane, 1,1,1-TCA was transformed. The interesting observation is that when phenol was pulse fed on day 56, 1,1,1-TCA transformation was observed. The transformation of 1,1,1-TCA was then observed on subsequent pulses of propane and phenol. The same result was achieved when a microcosm that was first stimulated on propane. Upon utilizing the phenol pulse (82 days), 1,1,1-TCA transformation was achieved (Figure 60). The results demonstrate that stimulation with propane resulted in 1,1,1-TCA transformation upon the addition of phenol. Note in Figure 58, when just phenol was fed into a microcosm, TCA was not transformed. We hypothesis that the propane-utilizers are actually responsible for 1,1,1-TCA transformation when phenol was fed. Propane-utilizers may be obtaining energy from the addition of phenol to this system, either likely from a phenol transformation product. This hypothesis is consistent with the long-term activity exhibited by the propane-utilizers and indicated that their enzyme system remains active for long periods in the absence of propane utilization.

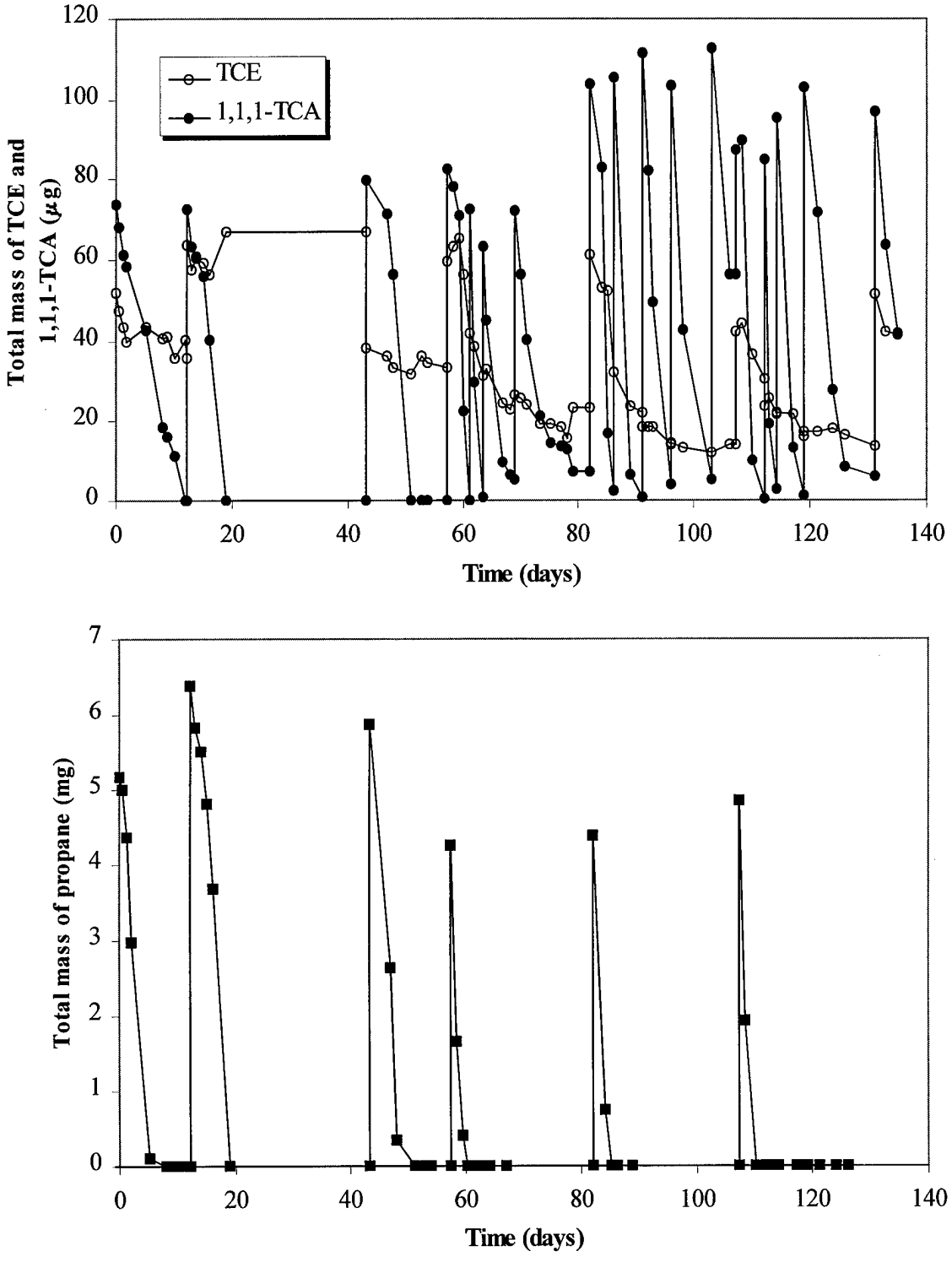


Figure 57. TCE and 1,1,1-TCA transformation in propane-fed microcosm PR#1

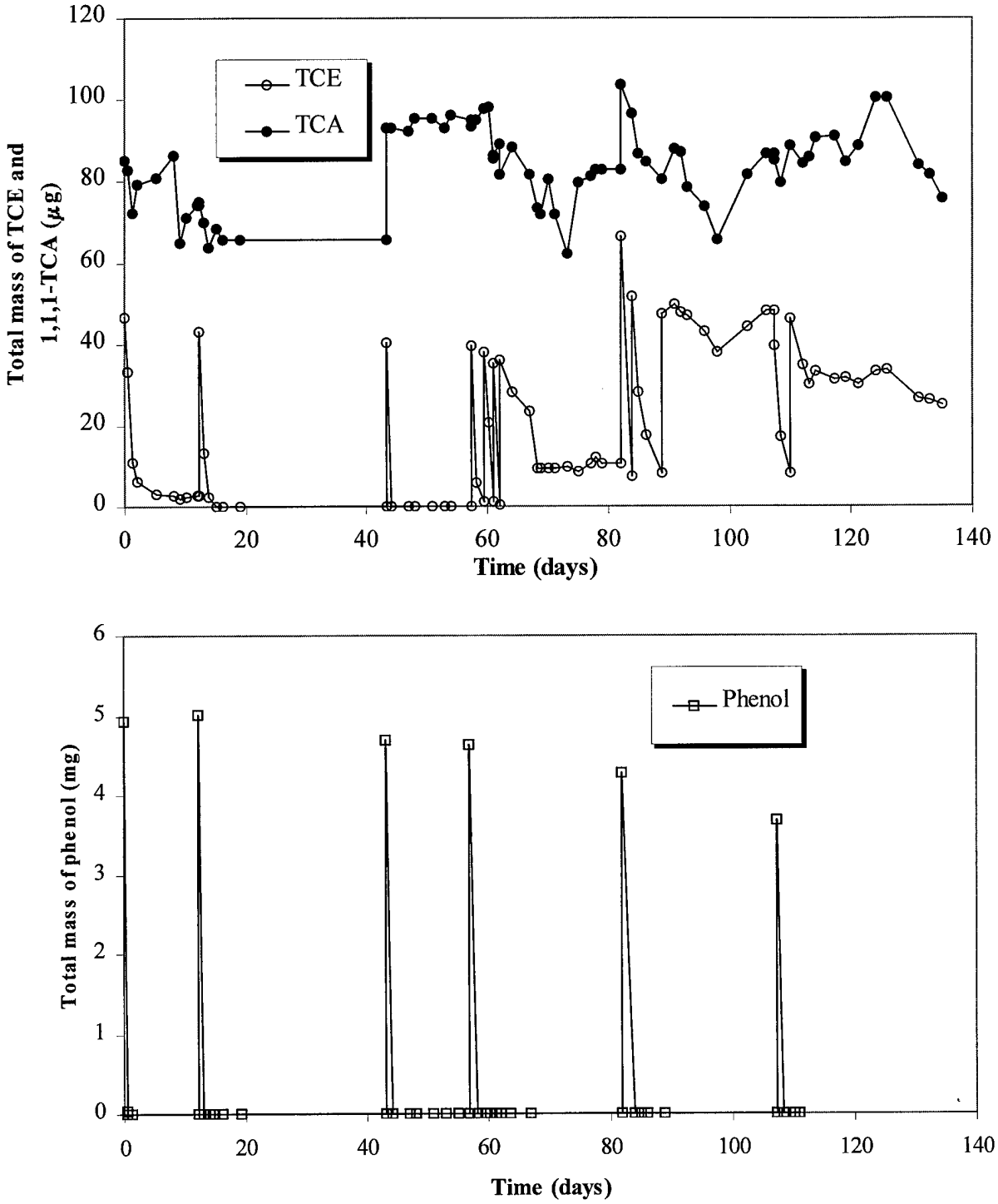


Figure 58. TCE and 1,1,1-TCA transformation in phenol-fed microcosm (Ph#1)

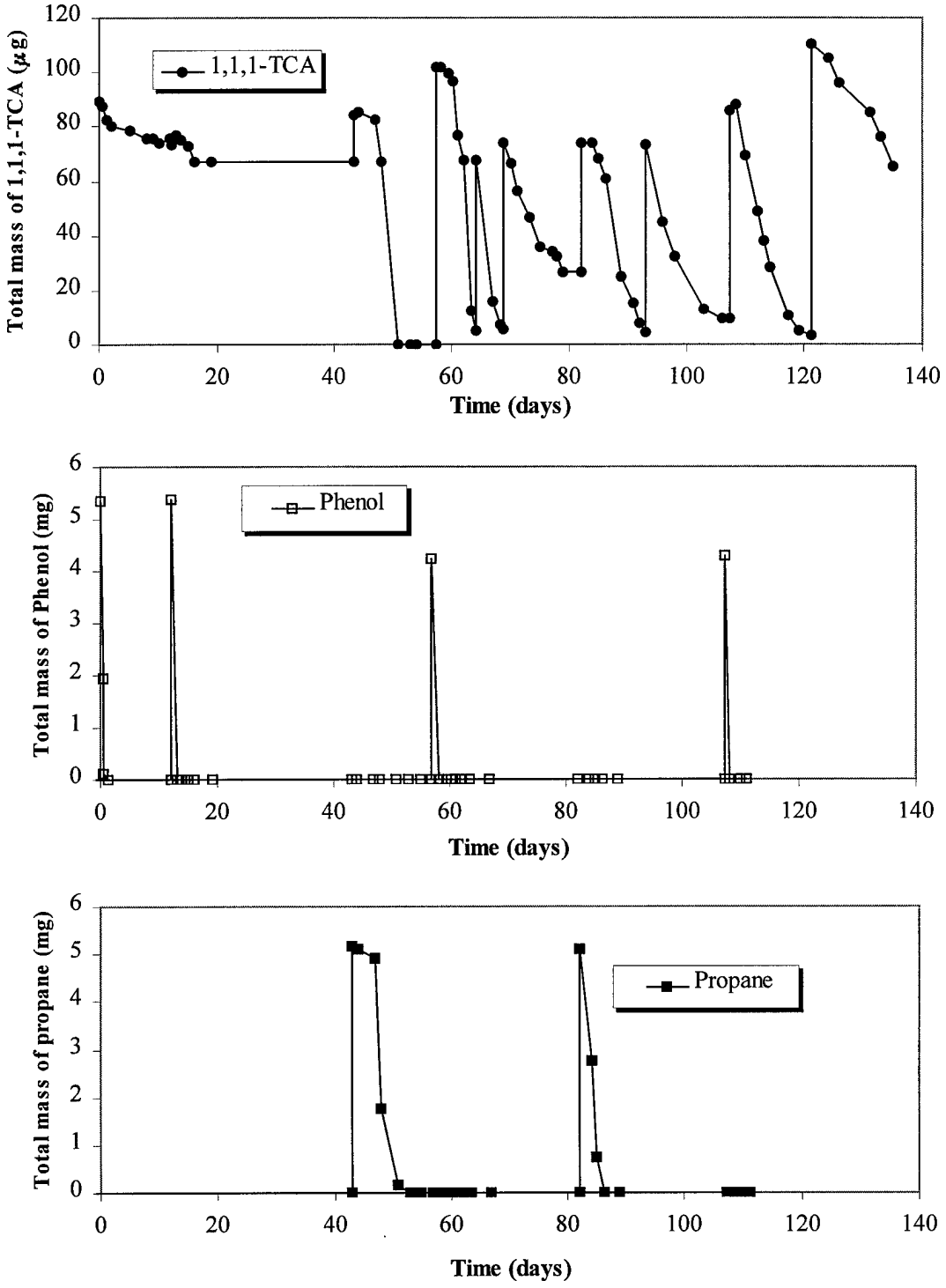


Figure 59. 1,1,1-TCA transformation using alternate propane and phenol pulsing in microcosm Ph#5

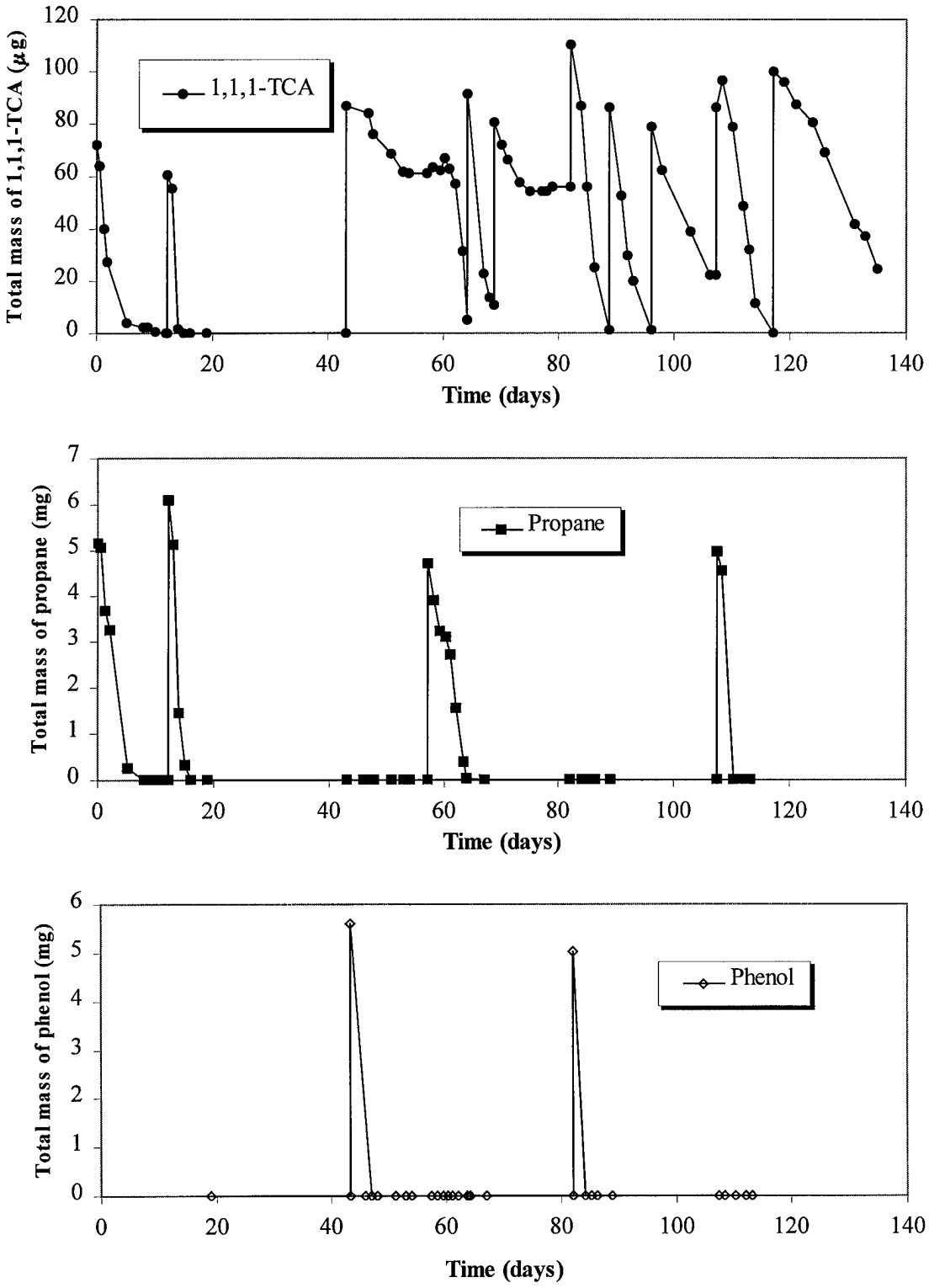


Figure 60. 1,1,1-TCA transformation using alternate propane and phenol pulsing in microcosm PR#5

The corresponding treatment for TCE alone is shown in Figures 61. In this case TCE was most effectively transformed during the phenol fed cycle. Limited TCE was transformed during the propane cycle, although slow prolonged transformation was achieved after propane was consumed (82 days). An interesting observation is that the phenol system remained active for a period of about 10 days after phenol was consumed. The results indicate that TCE transformation is not enhanced due to the alternate addition of mixed substrates. The enhancement transformation that was observed with 1,1,1-TCA upon switching to phenol was not observed with TCE upon switching to propane or phenol. Similar results were obtained with a microcosm that was first fed propane and then switched to phenol (Figure 62).

Figure 63 presents the results when phenol and propane were alternately pulse fed into a microcosm with both TCE and 1,1,1-TCA present. The microcosm was initially stimulated on propane, and effective 1,1,1-TCA, and limited TCE transformation was observed, consistent with the results presented in Figure 56. Upon switching to phenol, some TCE was transformed along with some 1,1,1-TCA. Again 1,1,1-TCA transformation was achieved upon switching to phenol. During the following cycles, 1,1,1-TCA was most effectively transformed during propane cycles, with some TCE transformed, and TCE was effectively transformed during phenol cycles, with some 1,1,1-TCA transformed. The most promising result is that 1,1,1-TCA was transformed along with TCE in a cycle, when phenol alone was fed.

Figure 64 presents the results for the 1,1,1-TCA/TCE mixture when the microbes were first stimulated on phenol. During the initial phenol cycle, TCE was transformed, but not 1,1,1-TCA, and in the initial propane cycle, 1,1,1-TCA was transformed. The next cycles with phenol (55 days) showed effective transformation of both TCE and 1,1,1-TCA. Transformation of both CAHs remained active for about 15 days after phenol was consumed. The following cycle with propane also showed TCE and 1,1,1-TCA being effectively transformed. The final pulse with phenol showed both TCE and 1,1,1-TCA being transformed. The duplicate-microcosm Ph2 (data not shown) showed the same trends, however, enhanced TCE and 1,1,1-TCA transformation was maintained during all the cycles after propane was added.

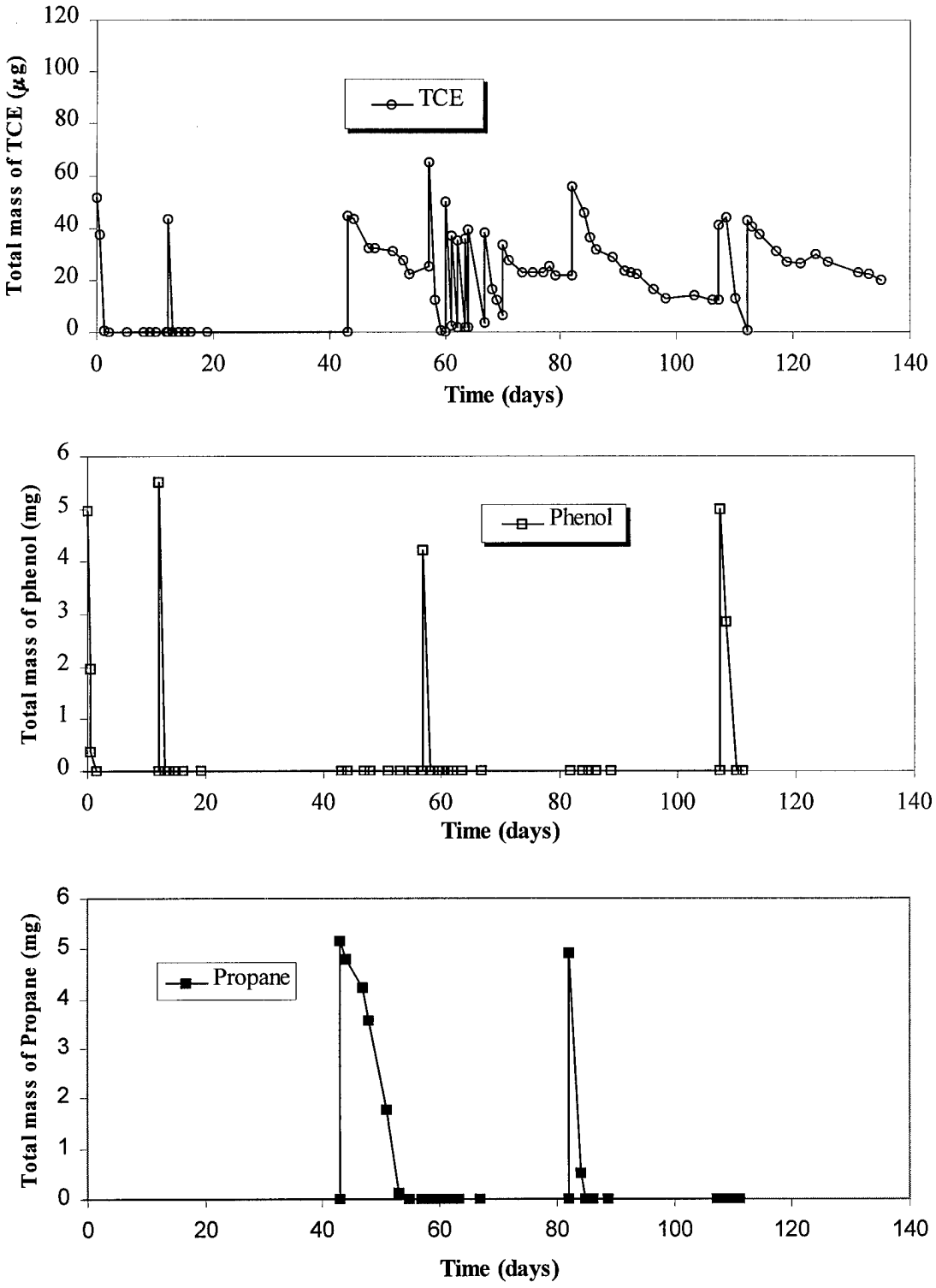


Figure 61. TCE transformation using alternate propane and phenol pulsing in microcosm Ph#4

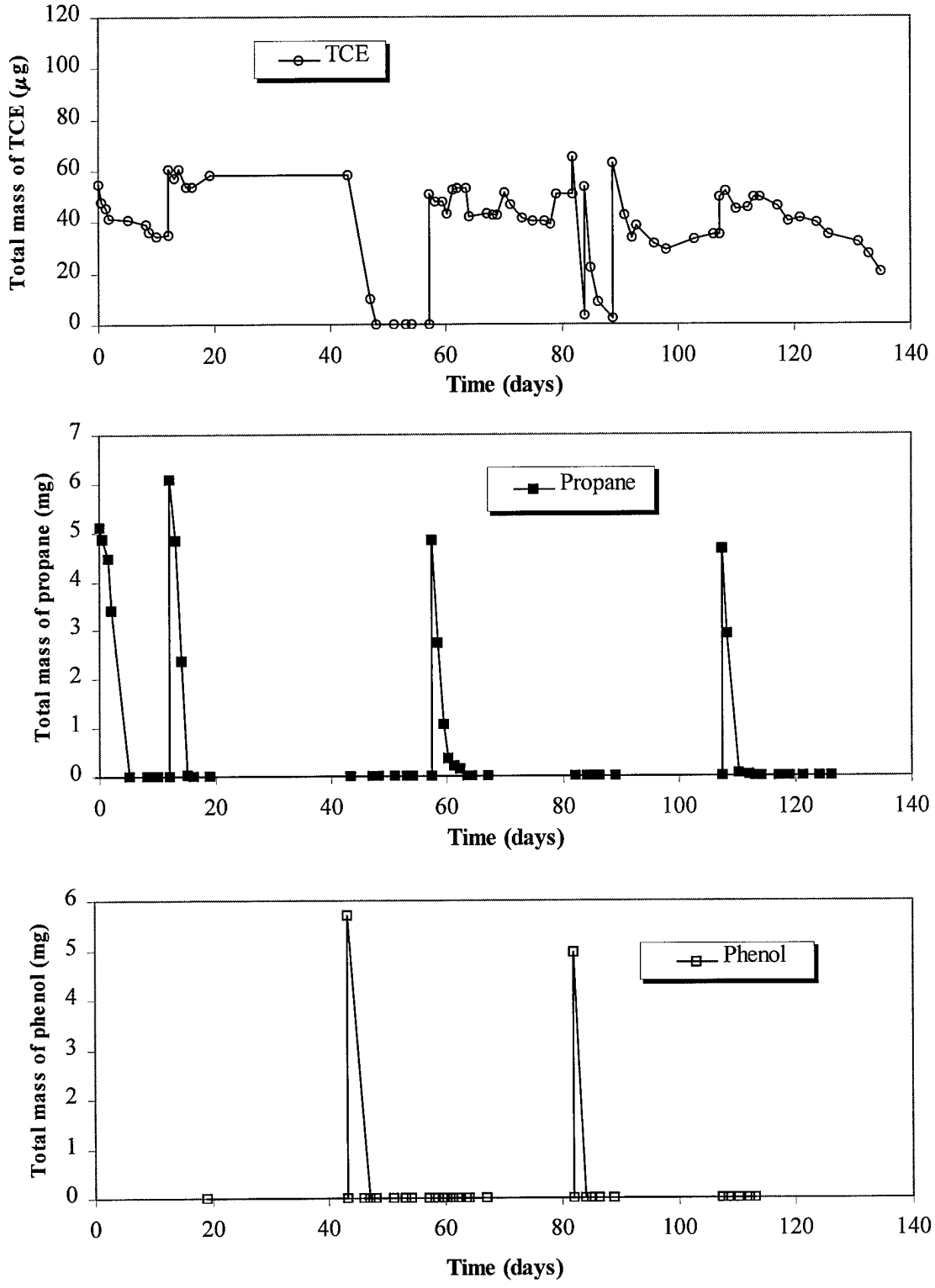


Figure 62. TCE transformation using alternate propane and phenol pulsing in microcosms PR#4

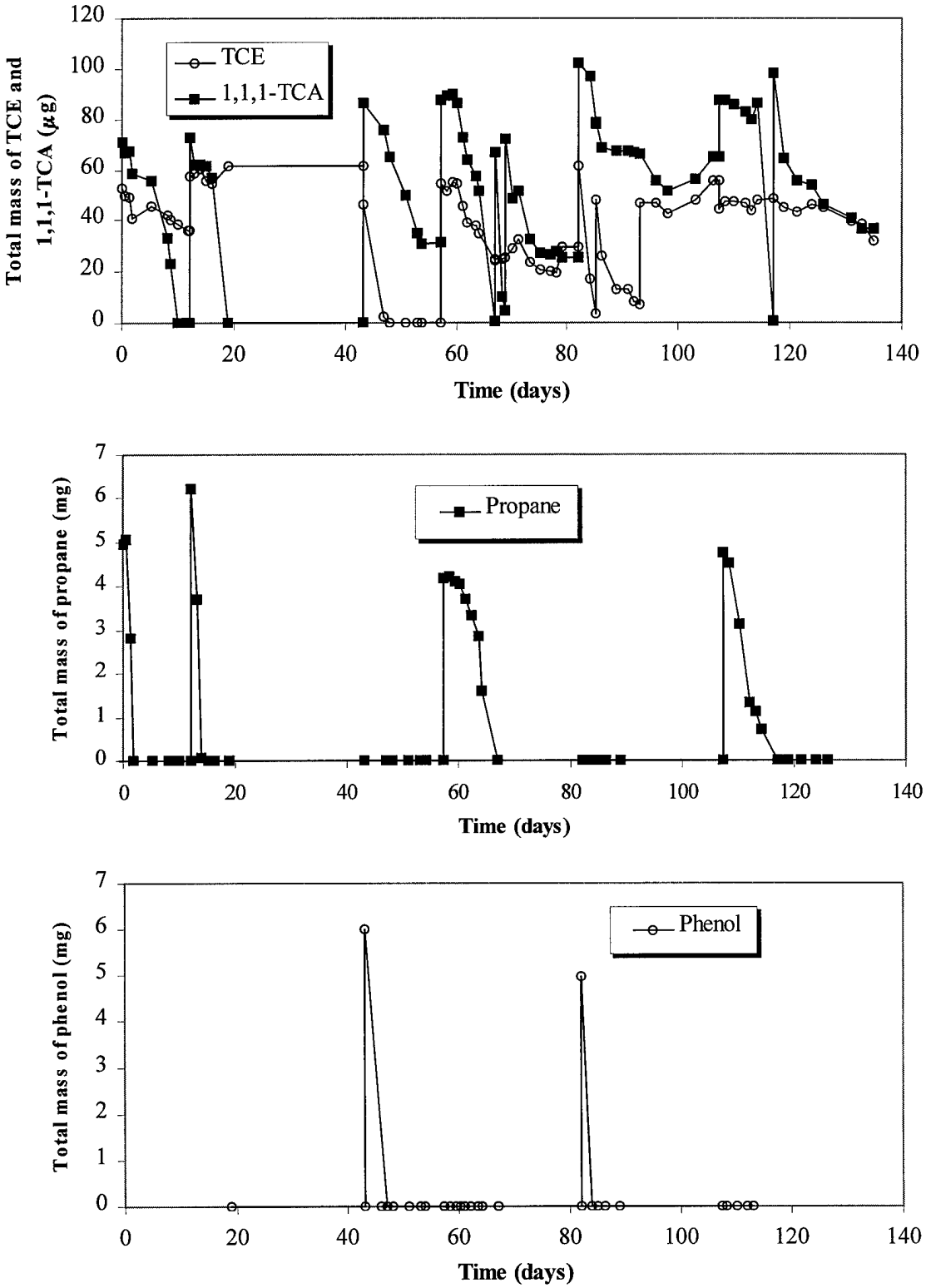


Figure 63. TCE and 1,1,1-TCA transformation using alternate propane and phenol pulsing in microcosm PR#2

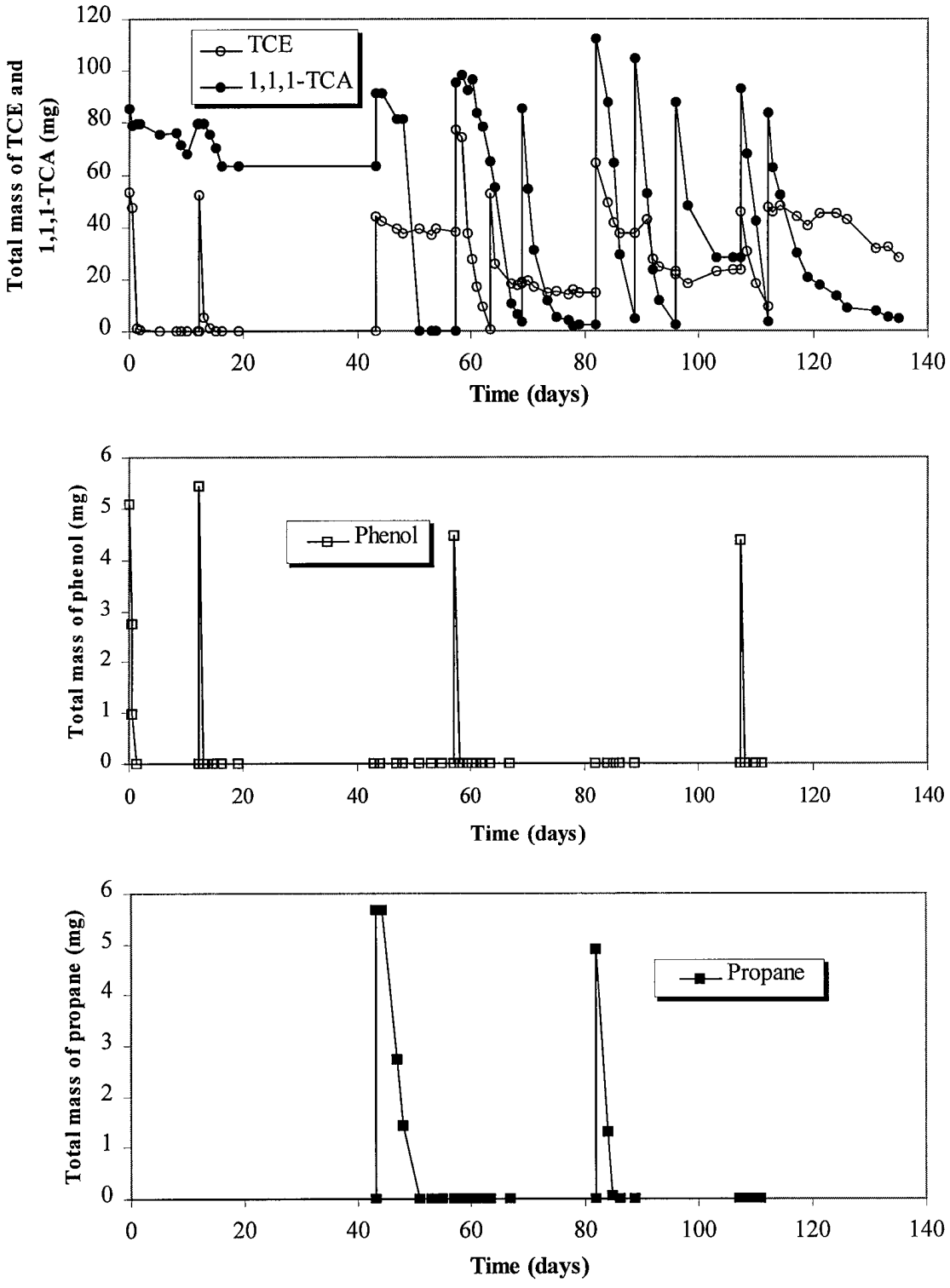


Figure 64. TCE and 1,1,1-TCA transformation using alternate propane and phenol pulsing in microcosm Ph#2

Presented in Figure 65 are the bar graphs that summarize the transformation yields obtained in the different treatments for the microcosms first stimulated on propane. PR1 was fed only propane throughout the course of this study, while challenged with both TCE and 1,1,1-TCA. This microcosm fed only propane in all the cycles were the most effective in transforming 1,1,1-TCA and some TCE transformation. Microcosms PR2 and PR3 were alternatively pulsed-fed with propane and phenol. TCE transformation slightly improved, while 1,1,1-TCA transformation decreased, compared to the microcosm fed only propane. Microcosms PR4 and PR5 were fed TCE and 1,1,1-TCA separately. It is interesting to note that TCE and 1,1,1-TCA transformation was similar to that achieved in the microcosm that contained both CAHs. Thus the presence of TCE and 1,1,1-TCA together did not appear to inhibit the performance of the microcosm-fed mixed substrates.

Presented in Figure 66 are the corresponding bar graphs for the microcosms first stimulated on phenol. Microcosm PH1, which was pulse fed only phenol, effectively transformed TCE, but showed no ability to transform 1,1,1-TCA, as previously discussed. Microcosms PH2 and PH3 were pulsed-fed with phenol and propane, and challenged with a mixture of 1,1,1-TCA and TCE. Effective transformation of 1,1,1-TCA and TCE was achieved. TCE transformation was almost as effective as that in the microcosm fed phenol alone. TCE was most effectively transformed during the alternate pulse feeding of phenol. In the phenol/propane pulsed microcosms that were challenged with the single CAH, the transformations were similar to that achieved when the mixtures of the CAHs were present. This observation is similar that obtained in the microcosms first stimulated on propane (Figure 65), and again indicate that the TCE and 1,1,1-TCA did not inhibit the transformation of the other CAH.

Comparing the results presented in Figures 65 and 66 shows that the stimulation with phenol first, (Figure 66) results in somewhat better transformation of the CAH mixtures than those stimulated with propane first. More detailed studies needed to determine if this is truly the case, and if so, what might be causing the difference to occur. It may be that different population of propane-utilizers results upon first stimulating the system on phenol, and then stimulating with propane.

The four transformation yields measured for each pulsing strategy (in Figures 65 and 66) were averaged to determine an overall transformation yield. Figure 67 compares the averaged TCE and averaged 1,1,1-TCA transformation yields using either a single substrate or alternating dual substrates. The averaged transformation yields of TCE and 1,1,1-TCA indicated that propane as a single substrate resulted in effective 1,1,1-TCA transformation and some TCE transformation, whereas phenol as a single substrate resulted only in TCE transformation. Pulsing with the dual substrates resulted in both TCE and 1,1,1-TCA transformations. The use of propane and phenol as mixed substrates was as effective for treating CAH mixtures as adding propane alone, with some improvement on the amount of TCE transformed.

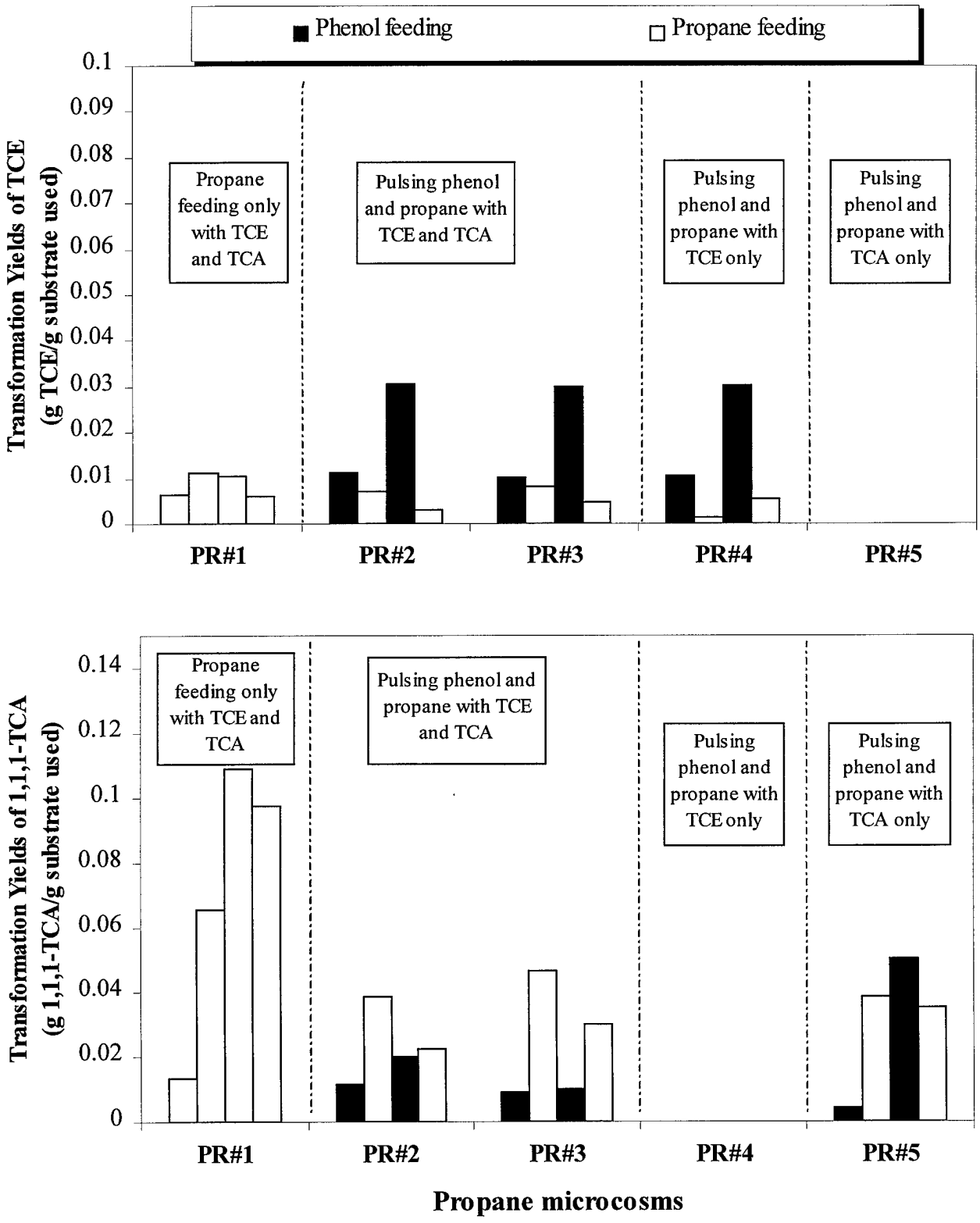


Figure 65. Comparison of TCE and 1,1,1-TCA transformation yields using a single (propane) or dual (propane and phenol) cometabolic growth substrate in microcosms initially stimulated on propane.

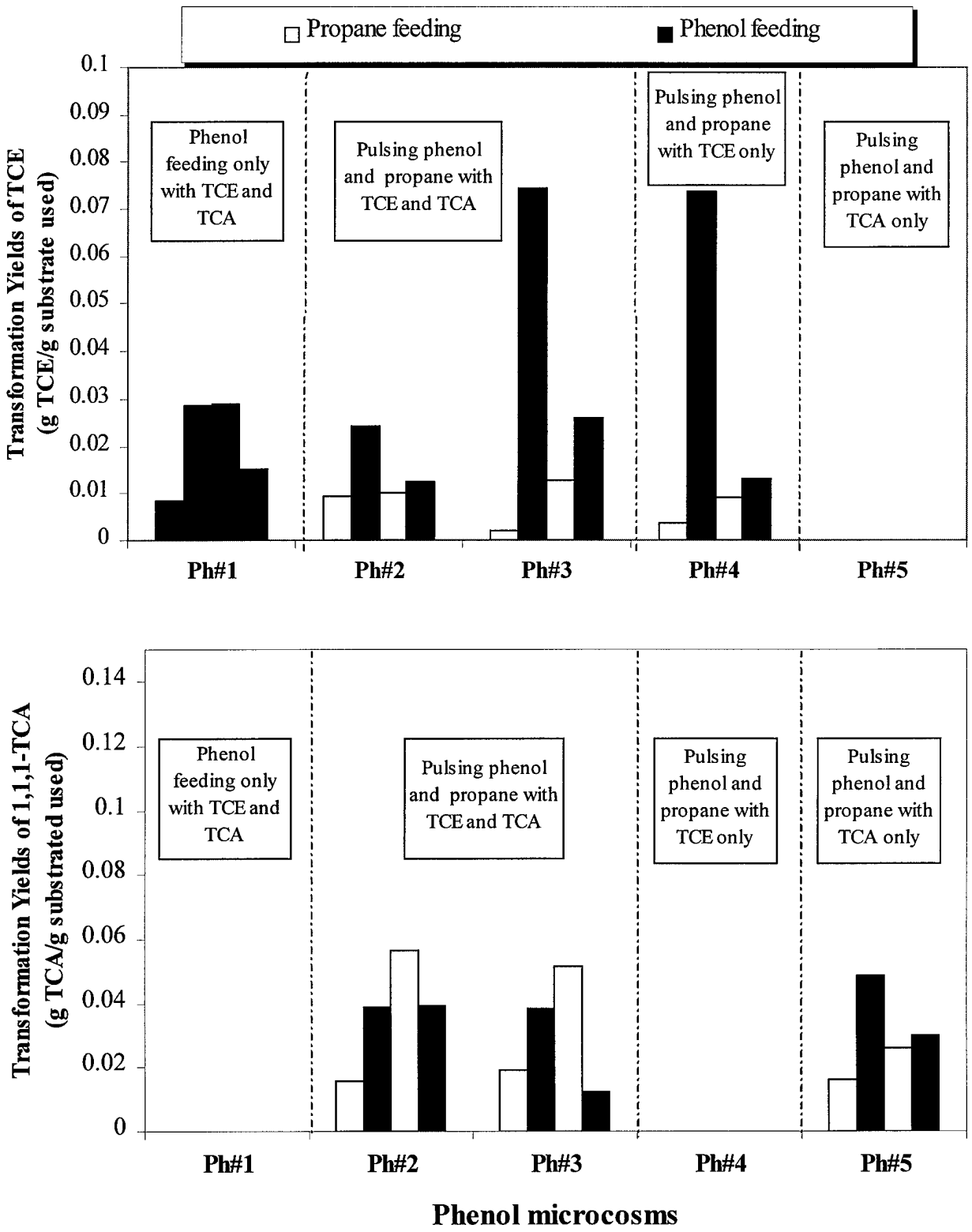


Figure 66. Comparison of TCE and 1,1,1-TCA transformation yields using a single (phenol) or dual (propane and phenol) cometabolic substrate in microcosms initially stimulated on phenol.

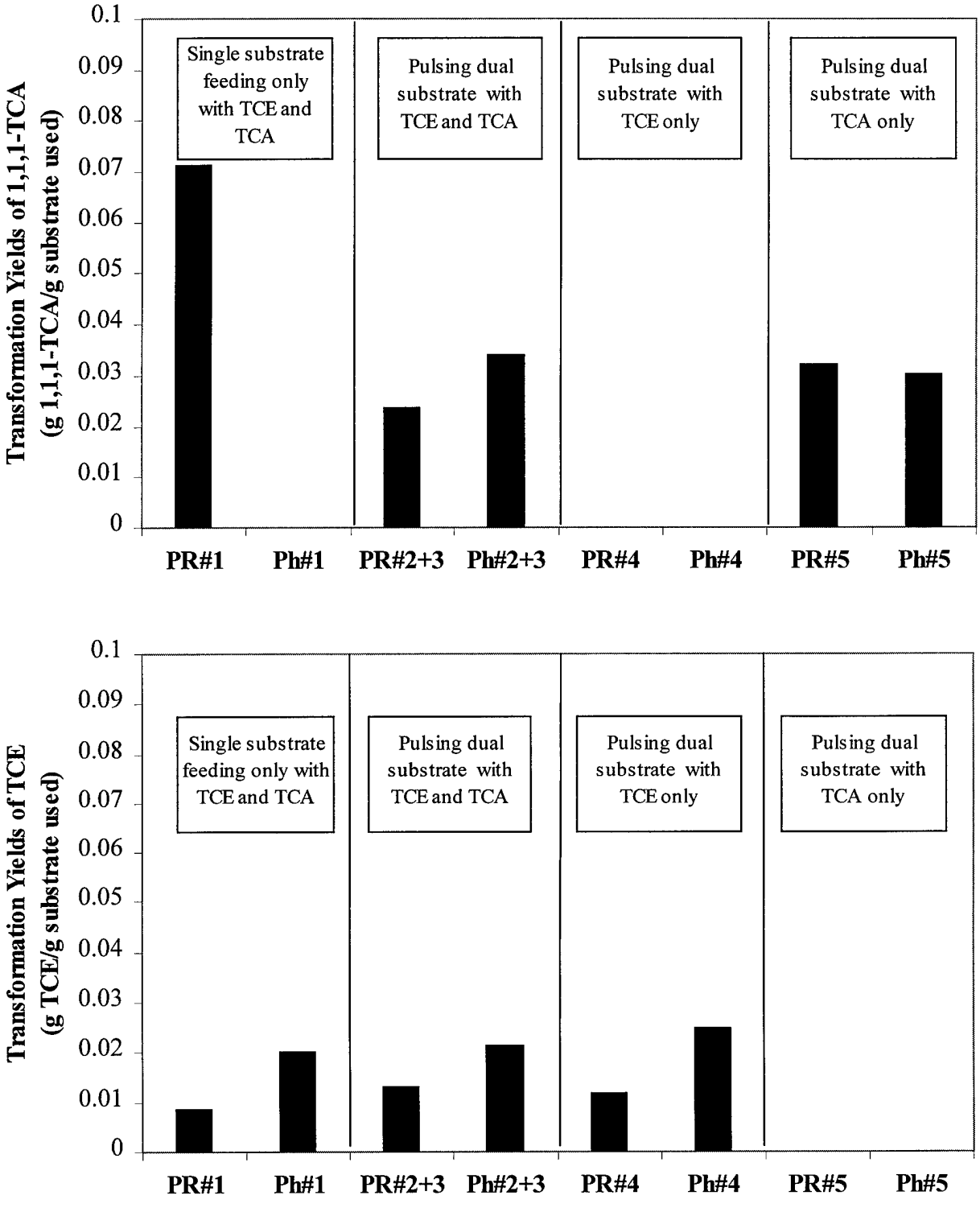


Figure 67. Comparison of averaged TCE and 1,1,1-TCA transformation yields using a single (propane or phenol) or dual (propane and phenol) cometabolic substrate.

Figure 68 shows the long-term transformation of 1,1,1-TCA resulting from the alternate pulsing propane and phenol into microcosm PR#5. This microcosm had been previously alternately pulse fed propane and phenol for 150 days (Figure 60). Propane was first fed to the microcosm followed by two additions of phenol. When propane was fed, effective 1,1,1-TCA transformation was observed. 1,1,1-TCA transformation continued upon phenol feeding as indicted from prior results. Transformation continued for 30 days after phenol was fed.

An unknown byproduct was also observed upon the addition of phenol. This product was observed on the high-pressure liquid chromatographic method that was used for phenol analysis with UV detection. The results indicate that the product was an aromatic compound resulting from the transformation of phenol. The ability to transform 1,1,1-TCA appears to be associated with the product formation from phenol addition. Long-term 1,1,1-TCA transformation was observed after phenol was consumed upon the second addition of phenol to the microcosm. Long-term 1,1,1-TCA transformation might be associated with phenol transformation that is induced by propane-utilizers. Phenol itself may induce propane-utilizers to transform 1,1,1-TCA or the unknown product may serve as an energy source for propane-utilizers.

Figure 69 shows TCE and 1,1,1-TCA transformation using propane as single substrate in microcosm PR#1. This microcosm had been previously fed propane as single substrate for 230 days {Figure 57 (the period of 0 to 140 days)}. The experiment was conducted over a short time period (hours) after 230 days of microcosm operation. Some TCE and effective 1,1,1-TCA transformation was observed after propane was consumed. No byproducts were observed in this microcosm.

TCE and 1,1,1-TCA transformation with the addition of phenol (after propane) is presented in Figure 70. This microcosm (PR#2) had been previously alternately pulsed fed with propane and phenol for 230 days before this experiment was conducted {Figure 63 (time period of 0 to 140 days)}. During the period of 140 to 230 days, 1,1,1-TCA was effectively transformed, and some TCE was transformed. Unknown byproducts were observed when phenol was pulsed fed into the microcosm. Phenol was rapidly utilized followed by TCE and 1,1,1-TCA transformation (Figure 70). Again, an unknown byproduct was observed upon adding phenol, which had the same retention time in the HPLC analysis as previous results. The maximum concentration of the unknown byproduct was observed when phenol degradation was nearly completed in the microcosm. The results indicate that phenol transformation likely results from the oxidation by propane-utilizers and the products formed may serve as energy sources to promote TCE and 1,1,1-TCA cometabolism.

Figure 71 shows TCE and 1,1,1-TCA transformations when pulsing of phenol after propane. Microcosm PR#3 had been previously operated as a duplicate to microcosm PR#2 prior to the experiment shown in Figure 71. TCE was effectively transformed and limited 1,1,1-TCA transformation was observed during the period of 140 to 230 days when phenol was pulsed fed (after propane). No byproducts were observed in the microcosm during that period. It is also interesting that effective TCE transformation was observed when byproducts were not produced. Figure 71 shows the results of an experiment after 230 days of stimulation with phenol and propane. A minimal amount of unknown byproduct accumulated in the microcosm during the period of phenol degradation. TCE was effectively transformed while minimal transformation of 1,1,1-TCA was observed. Thus, the lack of product formation appears to be associated with minimal 1,1,1-TCA transformation.

The results from Figures 70 and 71 indicate that the production of the unknown aromatic byproduct is associated with 1,1,1-TCA transformation. This hypothesis is supported by the results from microcosm fed phenol as single substrate. In this case, no 1,1,1-TCA transformation was observed and no byproducts were observed. The results indicate that propane-utilizers complete with phenol-utilizers for the phenol, and propane-utilizers get some benefit for cometabolism from the transformation of phenol.

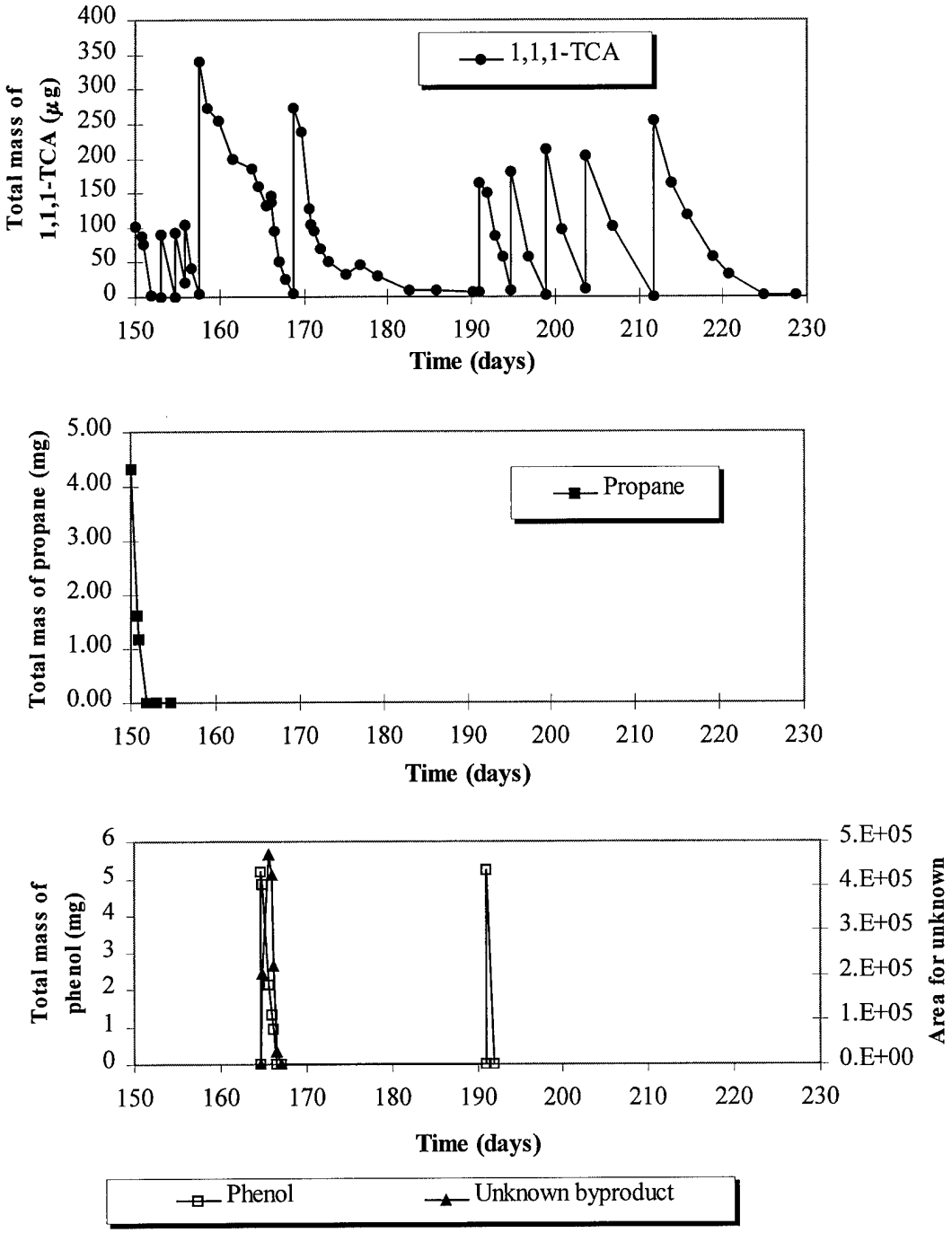


Figure 68 1,1,1-TCA transformation using propane and phenol pulsing in microcosm PR#5. Unknown byproducts resulting from phenol addition detected of HPLC analysis by UV detection are also presented.

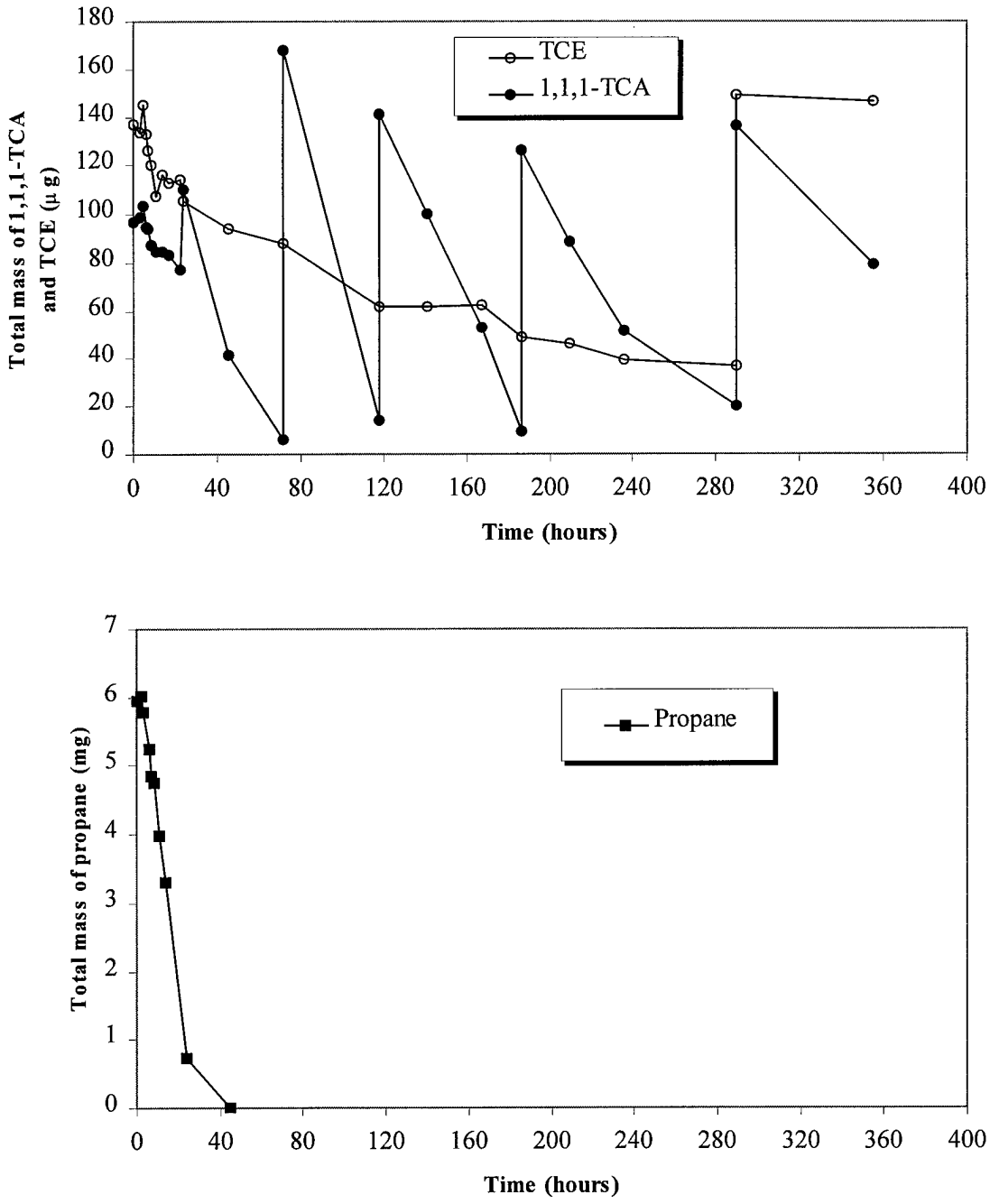


Figure 69 TCE and 1,1,1-TCA transformations using propane as single substrates in microcosm PR#1 in a short time periods (hours). The experiment was conducted over a short time period after 230 days in microcosm fed propane as single substrate.

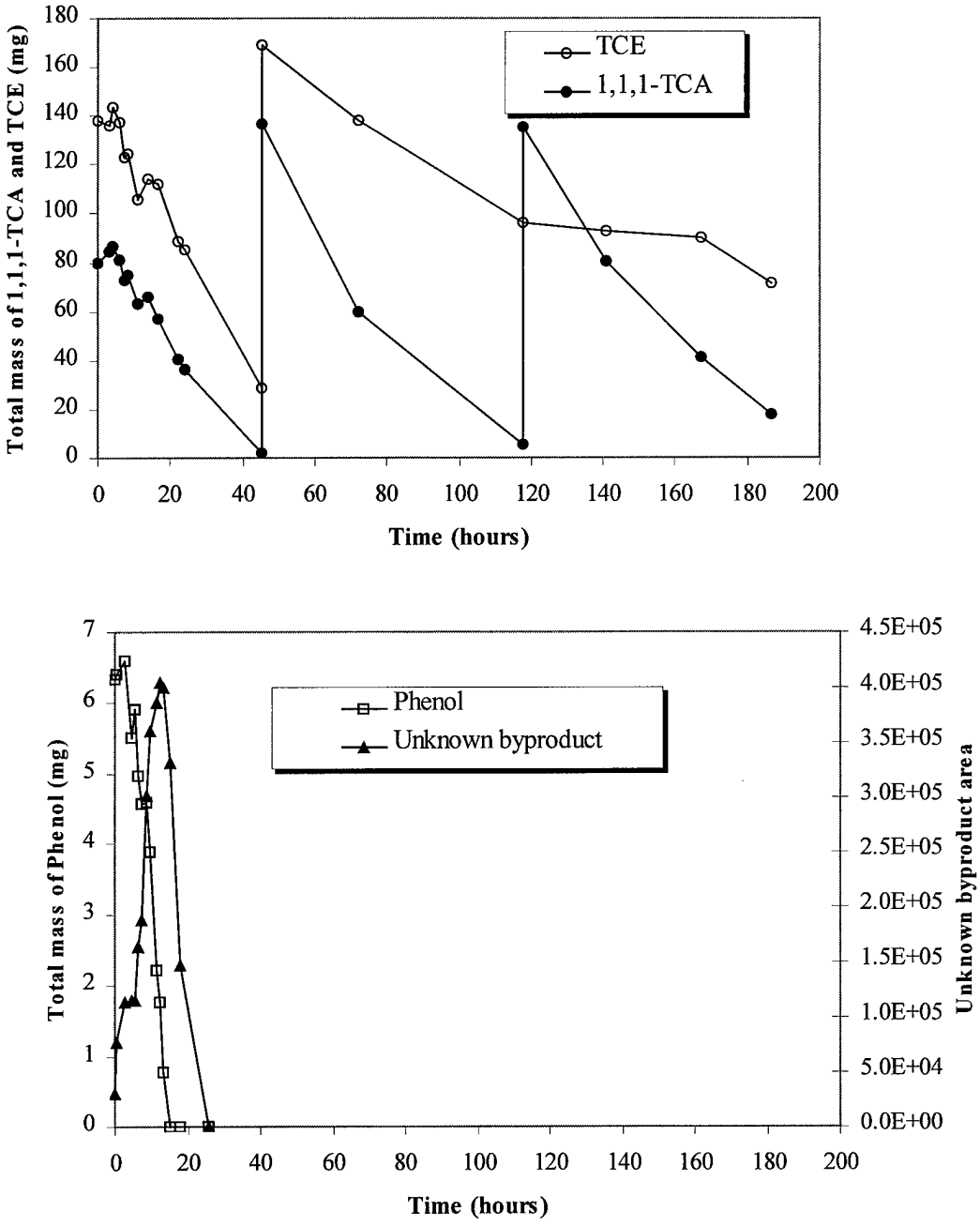


Figure 70 TCE and 1,1,1-TCA transformations over a short time periods (hours) when pulsing phenol after propane in microcosm PR#2. The unknown byproduct (GC peak area) is presented on second y-axis. The experiment was conducted after 230 days of alternate pulsing of phenol and propane.

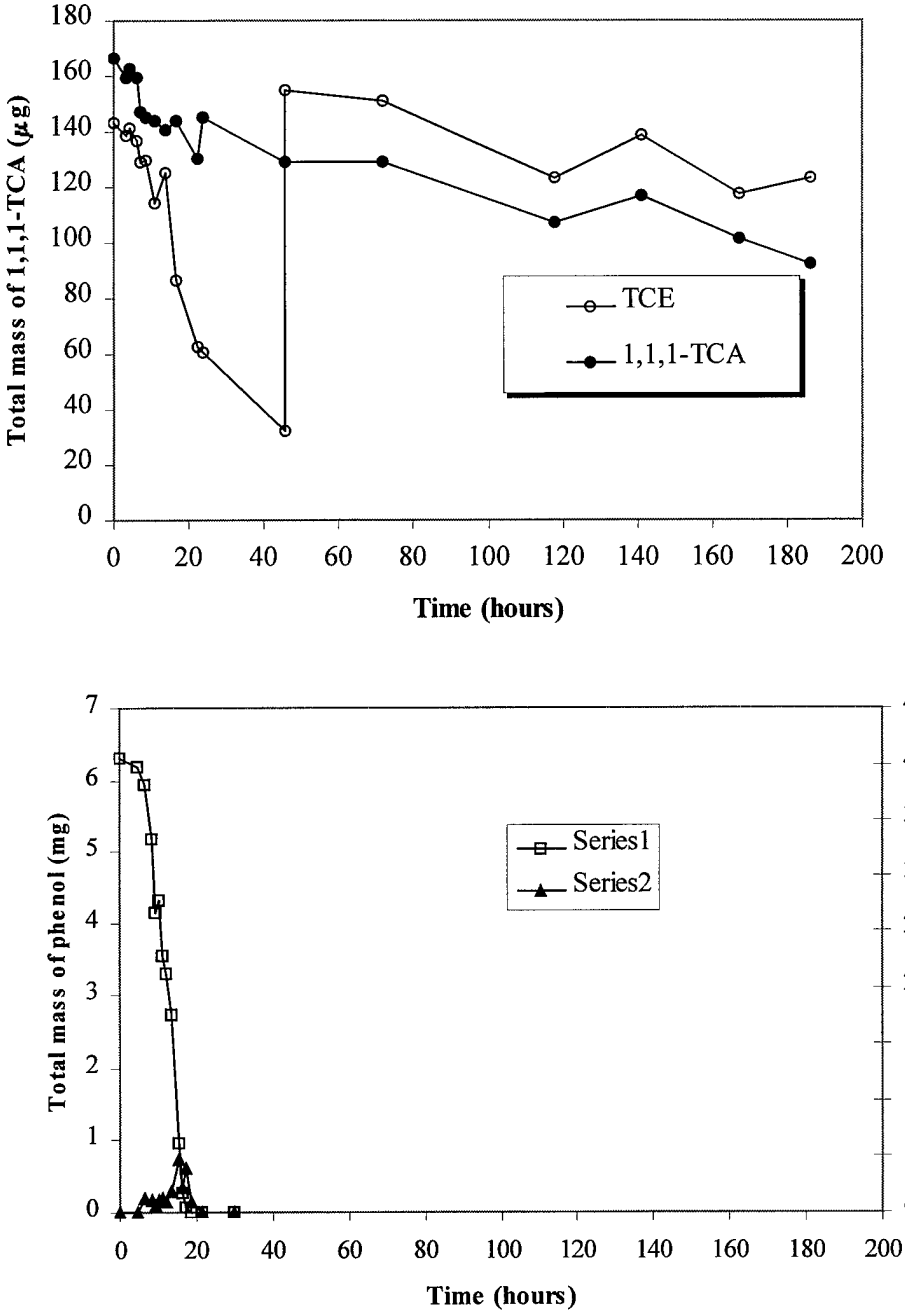


Figure 71 TCE and 1,1,1-TCA transformations when pulsing phenol after propane in microcosm PR#3. The unknown byproduct resulting from phenol addition (GC peak area) is presented on second y-axis. The experiment was conducted over short time period after 230 days of stimulation with phenol and propane.

In order to study the product formation in more detail, the microcosm used previously (in Figure 70) was split into two 65-ml serum microcosms. The two duplicate microcosms were labeled as PR#21 and PR#22. Phenol, TCE and 1,1,1-TCA were added both microcosms, and the microcosm were initially operated in the same manner. 30% propane was added to microcosm PR#22 after 3 hours of phenol transformation to determine if the presence of propane blocked the unknown product formation. Pure oxygen was added to maintain the requirement of oxygen in the microcosms.

Figure 72 shows TCE and 1,1,1-TCA transformation and phenol transformation in the microcosms PR#21 and PR#22. Phenol transformation and unknown byproduct formation was observed in both microcosms. The rates of phenol utilization and the rates of byproduct formation in both microcosms with or without propane were similar, indicating no effect of high propane concentration on phenol transformation and byproduct formation. TCE and 1,1,1-TCA were transformed in microcosm PR#21 after phenol and the byproducts were transformed, while microcosm PR#22 with 30% propane showed more limited TCE and 1,1,1-TCA transformation. This results suggest propane blocks the enzyme that responsible of TCE and 1,1,1-TCA transformations. However, inhibition of propane on phenol utilization and byproduct formation was not observed.

The reason why propane inhibited TCE and 1,1,1-TCA transformation, but it did not block phenol utilization or byproduct formation, is not known. There are several possible explanations. One possibility is that phenol has a lower K_s than propane, TCE, and 1,1,1-TCA, for enzyme that responsible to its transformation. The propane concentration in solution (20 mg/L) was likely not high enough to block the transformation of 110 mg/L of phenol in solution. TCE and 1,1,1-TCA concentration were over an order of magnitude lower in concentration than phenol. Thus, they were more effectively blocked. Another possible explanation is that the enzyme transforming phenol and producing byproducts may be different from the enzyme that responsible to propane utilization and TCE and 1,1,1-TCA transformation. More research is required to understand the mechanism of phenol transformation and to determine why byproduct formation seems to be associated with 1,1,1-TCA cometabolism.

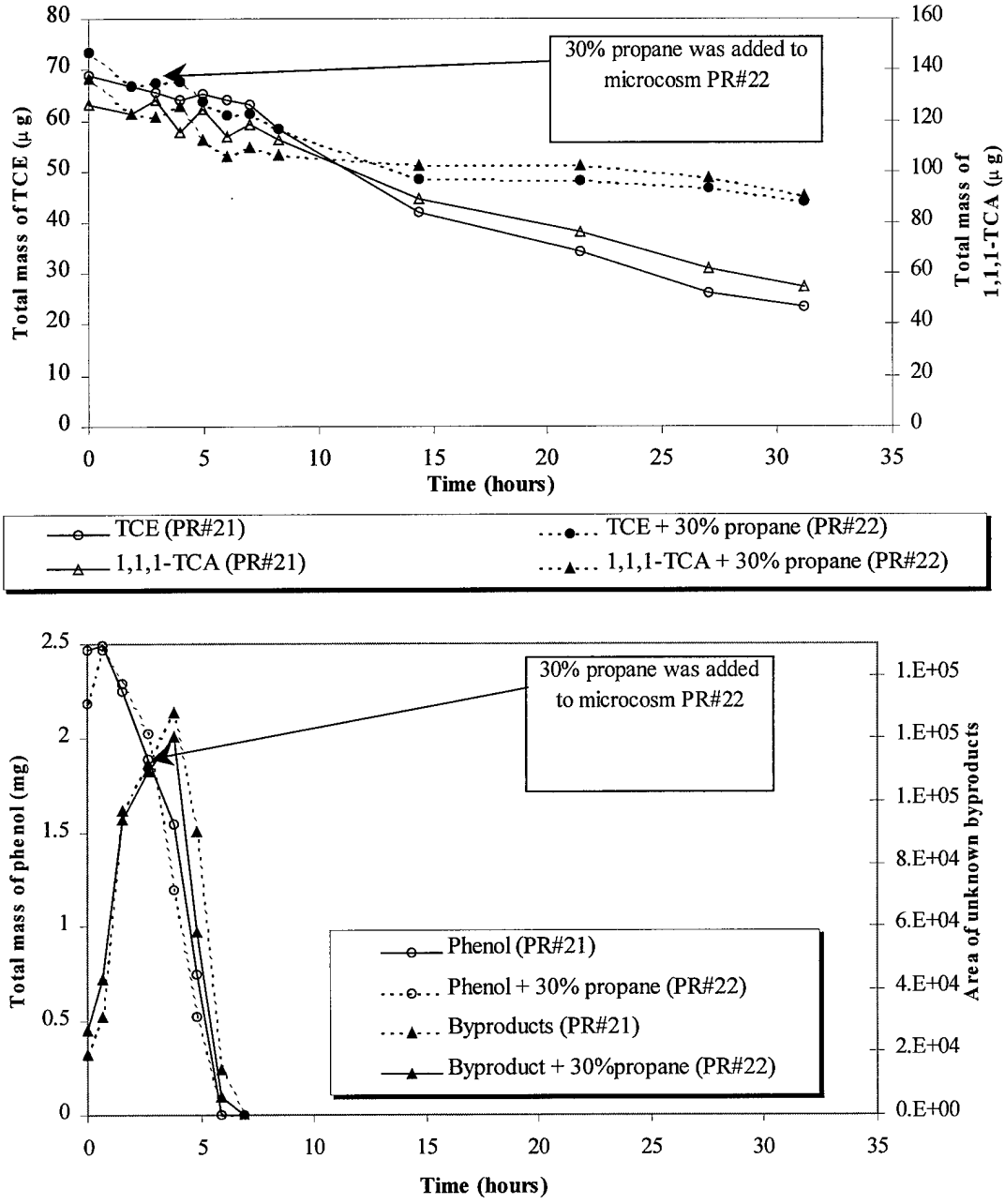


Figure 72 TCE and 1,1,1-TCA transformations in microcosm PR#21and PR#22. The peak areas for the unknown aromatic byproducts resulting from phenol addition is presented on second y-axis.

D. DISCUSSION

These initial experiments on the transformation of TCE/1,1,1-TCA mixtures with mixed- cometabolic substrates indicate that there is potential in this approach. Our results would indicate that the systems that may work best are those that work well for different CAHs. For example phenol-utilizers transform TCE well, but do not transform 1,1,1-TCA, while propane-utilizers effectively transform 1,1,1-TCA, but have limited abilities to transform TCE. When the dual substrates are used together, the ability to cometabolize both TCE and 1,1,1-TCA is realized. One exciting possibility is when the enzymatic systems involved in the cometabolism are very different, with one system used to initiate the oxidation of an aromatic and the other for a short chained-aliphatic. We initially hypothesized that the differences in these systems would likely result in limited competitive inhibition among the substrates. However, as we observed in the propane/phenol system, propane-utilizers likely competed with phenol-utilizers for phenol, which can be problematic.

The surprising observation is that the stimulation of propane-utilizers resulted in 1,1,1-TCA transformation when phenol was fed. We hypothesize that it was the propane-utilizers that were responsible for this transformation, and that energy derived from phenol or transformation products of phenol were driving the TCE and 1,1,1-TCA transformation. The long-term activity of the propane-utilizers towards CAH cometabolism in the absence of propane utilization supports this hypothesis. Since the enzymes remain active for long periods, endogenous energy sources might be used to drive the cometabolic process. More research is needed to test this hypothesis. This initial work would indicate that propane-utilizers would likely be a good choice for system with mixed-cometabolic substrates due to their long-term activity.

It is appears in the propane-phenol system that the propane-utilizers and the phenol-utilizers compete for phenol. Aromatic products are usually observed with phenol utilization, when some cometabolic transformation ability remaining with the propane-utilizers. Thus, the ability to maintain both propane-utilizers to degrade 1,1,1-TCA and phenol utilizers to transform TCE needs to be carefully balanced. Here, the substrates were alternately fed. Additional work needs to be performed where the substrates are both fed together, to determine if transformation can be enhanced. Products formed with phenol transformation need to be identified. We suspect that the products of this transformation are among ortho-, meta-, and para-catachol. Methanotrophs for example have been shown to produce ortho and para-catachol from phenol transformation.

Future work should focus on the propane/aromatic system with either phenol or toluene used as substrates along with propane. The microcosm studies which were initiated in this work should be continued. Work that needs to be completed is: 1) pulsing of both phenol and propane together in the microcosms. 2) identifying the by-products formed from phenol transformation. 3) determining if the products serve as alternative energy sources for the propane-utilizers. 4) determining if the same enzyme used for propane

utilization is required for phenol transformation. That work needs to be compared with the pulsing of phenol and propane as single substrates. An important comparison is whether with prolonged stimulation, the mixed phenol/propane system works better than the propane system alone, since the propane system alone can degrade both 1,1,1-TCA and TCE. The basis of comparison needs also to be considered. One basis would be the transformation yield basis, as presented here. The other would be on the amount of CAH transformed compared to the amount of oxygen consumed. Jenal-Wanner and McCarty (1997) indicated that oxygen consumption is important to consider due to the difficulties in adding dissolved oxygen in the field.

Future work should evaluate dual-cometabolic substrates in flow through systems with soil columns. The alternate pulsing of the substrates will likely be a key factor in such a system as well as the concentrations of the CAH mixtures. Studies should be initially performed with a mixture of two CAHs, such as TCE and 1,1,1-TCA, but should then be expanded to component mixtures that are representative of contaminated sites of interest to the Air Force. Toluene should also be evaluated, since the recent in-situ tests (McCarty et al., 1998) indicate that toluene might be easier to use in practice than phenol.

SECTION VII

CONCLUSIONS

1. The initial microcosm screening tests indicated indigenous methane, propane, and butane-utilizing microorganisms were present at in the subsurface of the McClellan AFB. Methane and propane-utilizers transformed TCE, however butuane-utilizers showed no TCE transformation ability. Methane-utilizers from the subsurface of Edwards AFB were stimulated that transformed TCE, but they were less effective than those observed at McClellan. Propane and butane-utilizers could not be stimulated in microcosms constructed with Edwards aquifer material and groundwater. With microcosms constructed with Moffett Air Field aquifer material and groundwater very long lag periods were encountered (80 to 90 days) for the stimulation of propane and butane-utilizers. The microcosms initially showed limited ability to transform 1,1,1-TCA.

2. Studies in Moffett Field microcosms focused on the bioagumentation of propane and butane-utilizing enrichments to reduce the growth lag period and to achieve effect 1,1,1-TCA transformation. Effective 1,1,1-TCA transformation was observed in the bioagumented microcosms, and also the unagumented microcosms over a period of one year. Successful bioagumentation required the addition of enrichments that worked well under the nutrient limited conditions of the groundwater. Effective enrichments for bioagumentation were obtained from the Moffett microcosms that had been operated for one year, where microorganisms were selected that effectively transformed 1,1,1-TCA under the nutrient limited conditions of the groundwater. PCR studies were found to be a useful means of studying population changes that occurred in the bioagumentation tests.

3. Indigenous methane-utilizers and propane-utilizers from the McClellan subsurface effectively transformed TCE for over a 1-year period. Methane-utilizers tolerated increases in TCE concentration better than propane-utilizers. The transformation yields were correlated with the ratios of the initial rates of TCE transformation to the initial rates of substrate utilization. Methane-utlizers transformed TCE most rapidly during the period of methane-utilization. Propane-utilizers showed prolonged transformation of TCE for up to four weeks after propane was consumed in the microcosms. Propane-utilizers were more effective at transforming CAH mixtures of CF, 1,1,1-TCA, and TCE, and were most effective in transforming 1,1,1-TCA. The methane-utilizers showed not ability to transform 1,1,1-TCA. Thus propane-utilizers are a good choice for the cometabolism of CAH mixtures at this site.

4. Nutrient tests indicated that the availability of fixed nitrogen was important for effective stimulation and transformation of TCE in McClellan microcosms. However with prolonged stimulation under nitrogen limited conditions very effective transformation of TCE was achieved in the methane stimulated microcosms. The results indicate that methane-utilizers that were able to fix nitrogen were stimulated in the microcosms. Propane-utilizers also grew under nitrogen-limited conditions, but enhanced TCE transformation was not achieved. Methane enrichments were obtained from the microcosm

that grew most rapidly in media that lacked nitrogen. The results indicate that the most effective methane-utlizers that might be obtained for remediation are those grown under nitrogen limited conditions.

5. Studies using mixed cometabolic substrates to transform CAH mixtures found consortiums grown on phenol and propane were more effective in transforming TCE and 1,1,1-TCA than those grown on propane and methane. Phenol-utilzers from McClellan AFB could transform TCE, but showed no ability to transform 1,1,1-TCA. Propane-utilizers effectively transformed 1,1,1-TCA and showed limited ability to transform TCE. With the alternate addition of propane and phenol to the microcosms, 1,1,1-TCA and TCE was transformed upon the addition of phenol. The ability to transform 1,1,1-TCA when phenol was added corresponded to the formation of aromatic by-products from phenol-utilization. The results indicate that propane-utilizers were competing with phenol-utilizers for phenol and gained benefit from the transformation of phenol to cometabolize 1,1,1-TCA. When phenol was added the presence of high propane concentrations, the presence of propane blocked 1,1,1-TCA and TCE transformation, indicating the same enzyme for propane utilization was required for these transformations. More effective transformation of 1,1,1-TCA and TCE mixtures was achieved through the alternate addition of phenol and propane than by the alternate addition of propane alone. The results are encouraging for the transformation of CAH mixtures using propane and phenol, and possibly propane and toluene, as mixed cometabolic substrates.

SECTION VIII

RECOMMENDATIONS

Bioaugmentation is a promising method for the addition of enrichments of propane and butane-utilizing microorganisms for in-situ bioremediation. Based on our screening studies propane and butane-utilizers are not ubiquitous in the subsurface, and if present, often require long lag periods to stimulate. Butane-utilizers, that are present, often show little CAH transformation ability, compared to laboratory enrichments. More work is needed to determine conditions required for successful bioagumentation. Our work indicates that most successful approach would be to add enrichments that work well under the ambient nutrient conditions at the site. Future works should include: determining the factors that effect successful bioaugmentation over a range of subsurface conditions at DOD sites; determining the most effective means of growing the enrichments while maintaining their abilities to perform well under subsurface nutrient limited conditions; evaluating bioaugmentation addition strategies; and developing molecular methods for tracking the bioagumented cultures. With this additional work, a field trial of the bioagumentation concept should be performed, with the Moffett test site, the likely candidate site for this trial.

Indigenous propane-utilizers are excellent candidates for the cometabolism of CAH mixtures in the McClellan subsurface. More work is needed to determine the factors that cause the long-term activity of the propane-utilizers after propane is consumed. Isolation of pure cultures from the McClellan microcosms should be conducted to help in this determination. Work should also be performed with pure cultures from the ATCC culture collection for comparison. Propane-utilizers from other sites should also be studied for their long-term transformation abilities.

A field study should be performed to test the ability to cometabolize CAH mixtures in to subsurface of McClellan AFB. A potential remediation scheme would be to sparge propane into the saturated zone to achieve cometabolism in both the saturated and unsaturated zone. The subsurface has a thick vadose zone (about 100 ft), thus sparging and treatment in both the saturated and unsaturated zones maybe a good approach for this site. Methods that take advantage of the long-term activity of TCE transformation observed with propane-utilizers should be considered for the creation of passive barriers for bioremediation.

The use of phenol and propane, and possibly toluene and propane, as mixed cometabolic substrates, to transform CAH mixtures should be investigated in more detail. Along with mixed cultures, studies with pure cultures should be performed. Population dynamics should be studied in more detail, to determine the distribution and activity of the populations. More information is also needed at the enzyme level on the competitive inhibition among substrates. The methodology for dual substrate addition for stimulation appears to be critical for the maintenance of effective populations to transform the CAH mixtures. Pulsing strategies (alternating versus adding the substrates together) should be

investigated. The amounts of differ substrates added should be investigated as well as the timing for their addition. Continuous flow column studies should be performed to optimize the process. A field demonstration of the dual substrate addition method should be performed after more information is obtained on the process.

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